FORM PTO-1390 (Modified) (REV 5-93) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		DALHO1340-1
		U S APPLICATION NO (If known, see 37 C F R 1 9) Unknown 10/019453
INTERNATIONAL APPLICATION NO. PCT/US00/10954	INTERNATIONAL FILING DATE 21 April 2000	PRIORITY DATE CLAIMED 23 April 1999
TITLE OF INVENTION GENETICALLY ENGINEERED ORAL COMMENSAL ORGANISMS AS VACCINES		
APPLICANT(S) FOR DO/EO/US Song F. Lee and Scott A. Halperin		

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   - ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   - ☐ has been transmitted by the International Bureau.
   - ☒ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   - ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   - ☐ have been transmitted by the International Bureau.
   - ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   - ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).
11. ☒ Applicant claims small entity status under 37 CFR 1.27 .

Items 12. to 17. below concern other document(s) or information included:

12. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
13. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
14. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☒ A change of power of attorney and/or address letter.
17. ☒ Other items or information: Postcard

U.S. APPLICATION NO (If known, see 37 C.F.R. 1.50)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
10/019453	PCT/US00/10954	DALHO1340-1

18. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5): Search Report has been prepared by the EPO or JPO $890.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) $710.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) $740.00		
Neither international preliminary examination fee (37 CFR 1.482) nor International search fee (37 CFR 1.445(a)(2)) paid to USPTO $1,040.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) $100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$710.00	
Surcharge of $130.00 for furnishing the oath or declaration later than 20 Months from the earliest claimed priority date (37 CFR 1.492(e))	$130.00	

Claims	Number Filed		Included in Basic Fee		Extra Claims		Rate		
Total Claims	35	-	20	=	15	×	$18.00	$270.00	
Independent Claims	1	-	3	=	0	×	$84.00	$0.00	
Multiple dependent claim(s) (if applicable)							$280.00		
TOTAL OF ABOVE CALCULATIONS =								$1,110.00	
Reduction by ½ for filing by small entity, if applicable.								$555.00	
SUBTOTAL =								$555.00	
Processing fee of $130.00 for furnishing English translation later the 20 months from the earliest claimed priority date (37 CFR 1.492(f). +									
TOTAL NATIONAL FEE =								$555.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +									
TOTAL FEES ENCLOSED =								$555.00	

				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of $555.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 50-0872 in the amount of $_____ to the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-0872. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
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SIGNATURE

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REGISTRATION NUMBER 31,192

DATE 23 OCTOBER 2001

Atty. Dkt. No. DALHO1340-1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Lee and Halperin

Title: GENETICALLY ENGINEERED ORAL COMMENSAL ORGANISMS AS VACCINES

Appl. No.: Unknown

Filing Date: 23 October 2001

Examiner:

Art Unit:

CHANGE OF CORRESPONDENCE ADDRESS

Commissioner for Patents
Washington, D.C. 20231

Sir:

Applicant's attorney respectfully requests that the records of the United States Patent and Trademark Office in connection with the above-identified application be changed to show the following address and telephone number for all future communications.

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Respectfully submitted,

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DOCUMENT: Transmittal Letter to the United States Designated/Elected Office (DO/EO/US) Concerning a filing under 35 U.S.C. 371 With Request for Change of Correspondence Address
DOCKET NO.: DALHO1340-1
SERIAL NO. Based on PCT/US00/10954

CERTIFICATE OF MAILING BY EXPRESS MAIL

EXPRESS MAIL MAILING LABEL NUMBER: EL796239108US

DATE OF DEPOSIT: 23 October 2001

I HEREBY CERTIFY THAT THIS PAPER OR FEE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE "EXPRESS MAIL POST OFFICE TO ADDRESSEE" SERVICE UNDER 37 CFR 1.10 ON THE DATE INDICATED ABOVE AND IS ADDRESSED TO THE ASSISTANT COMMISSIONER FOR PATENTS, WASHINGTON, D.C. 20231, BOX PCT.

Ruth Sabula
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Ruth Sabula
Signature

GENETICALLY ENGINEERED ORAL COMMENSAL ORGANISMS AS VACCINES

FIELD OF THE INVENTION

The present invention relates to compositions, such as vaccines, for the stimulation of immune protection against infection by a pathogen. In a particular aspect, the present invention relates to genetically engineered live oral commensal organisms for the stimulation of immune protection against infection by one or more pathogens.

BACKGROUND OF THE INVENTION

Mucosal pathogens are those that colonize oral/respiratory mucosa prior to infection and disease. These pathogens are most effectively combated by a strong mucosal immune response in the host, but the mucosal response in mammals has a short memory, necessitating repeated booster immunizations. A prime example is pertussis (whooping cough), a highly contagious disease of the respiratory tract which affects all ages, but has the greatest morbidity and mortality in young children. In 1995, the disease killed 355,000 children and affected 40 million people worldwide (World Health Organization, 1996). In North America and Europe, the number of reported cases of pertussis infection has increased over the last few years (N. Wortis et al., *Pediatrics* 97:607-612, 1996; S. Schmitt-Grohe et al., *Clin. Infect. Dis.* 21:860-866, 1995). Most of the cases are found in individuals who have not been vaccinated against pertussis. In Third World countries, the lack of an infrastructure to deliver the vaccine to the general population appears to be the major reason for the failure of vaccination.

The conventional pertussis vaccine is a suspension of inactivated *B. pertussis* whole cells as a part of the trivalent diphtheria-tetanus-pertussis vaccine (DTP) administered during the childhood immunization regimen. Significant local reactions (soreness), fever, and rare cases of severe adverse effects (brain damage and death), have been concerns for the whole-cell vaccine. The development and use of acellular

vaccines composed of various combinations of purified *B. pertussis* antigens (pertussis toxin, filamentous hemagglutinin, pertactin, and fimbriae 2 and 3) have eliminated some of the adverse effects (M.D. Decker and M. Edwards, *J. Infect. Dis.* 174 (Suppl 3):S270-275, 1996; H. Bernstein et al., *Vaccine* 13:1631-1635, 1995). However, the vaccines are still administered by injection, and a number of boosters are needed to achieve a high level of immune response. Unlike natural infection by *B. pertussis*, immunization with whole-cell or acellular pertussis vaccines does not reliably confer long-term immunity to *B. pertussis* infection.

Hence, milder cases of *B. pertussis* infection may be present among adolescents and adults whose immune response to pertussis has waned because they have not been boosted since childhood (J.D. Cherry, *J. Infect. Dis.* 174:(Suppl 3) S259-263, 1996; S.W. Wright et al., *JAMA* 273:1044-1046, 1995). These individuals may help to maintain and spread the disease to the most-vulnerable populations, such as infants (American Society for Microbiology, *ASM News* 62:518-519, 1996; *Morb. Mortal. Wkly Rep.*, 44:74-76, 1995). To overcome this problem, immunization programs with acellular pertussis vaccine may need to be introduced for older children and adults, additionally increasing the cost of health care. Even if the economy can adsorb this extra-cost, the problem of delivering the vaccine to people living in developing and under-developed countries, who have the utmost need of vaccination, remains unsolved. In fact, the use of acellular pertussis vaccines, even for childhood immunization, is still beyond the financial reach of most of the developing world.

B. pertussis, the causative agent of pertussis, is a small Gram-negative coccobacillus that spreads by aerosols and direct contact. The disease commences as a mild upper respiratory infection and progresses to episodes of paroxysmal coughing and vomiting. The first step in infection is bacterial adherence to ciliated cells of the respiratory tract. *B. pertussis* produces a number of adhesins, including filamentous hemagglutinin (FHA), pertactin, and fimbriae, for this purpose. FHA is a 220 kDa protein with multiple domains for interaction with carbohydrate and sulfated groups on lipids and proteins on cells of the respiratory tract (J.H. Hannah et al., *Infect. Immun.* 62:5010-5019, 1994; C. Locht, et al., *Mol. Microbiol.* 9:653-660, 1993; M.J.

Brennan et al., *J. Biol. Chem.* 266:18827-18831, 1991). Pertactin, a 69 kDa outer membrane protein, contains the RGD tripeptide motif for adherence (E.M. Leininger et al., *Proc. Nat'l. Acad. Sci. USA* 88:345-349, 1991), although a pertactin-negative mutant colonizes the trachea and lungs of mice as well as the wild-type (M. Roberts et al., *Mol. Microbiol.* 5:1393-1404, 1991). The types 2 and 3 fimbriae can bind sulfated sugars and integrin VLA-5, suggesting their role in mediating adherence to host cells (W.L.W. Hazenbos et al., *Infect. Dis.* 171:924-929, 1995; C.A. Geuijen et al., *Infect. Immun.* 64:2657-2665, 1996, C.A. Geuijen et al., 1997, *supra.*).

In addition to these adhesins, *B. pertussis* also produces a major ADP-ribosylating toxin, pertussis toxin (PT), which is responsible for intoxication and cellular dysfunction (J.J. Munoz, In R. D. Sekura et al. (eds), *Pertussis toxin*, Academic Press, New York, 1-18, 1985). Another toxin, adenylate cyclase toxin (ACase), which also elevates the intracellular cyclic AMP level leading to cellular dysfunction, is also produced by the bacterium. Cell-associated forms of PT and ACase are involved in adherence as well (E. Tuomanen et al., *Dev. Biol. Stand.* 61:197-204, 1986; A.A. Weiss et al., *Infect. Immun.* 42:33-31, 1983; M.S.M. Goodwin and A.A. Weiss, *Infect. Immun.* 58:3445-3447, 1990). Among these virulence factors, PT is clearly one of the most important as disruption of the PT genes renders the organism essentially avirulent (A.A. Weiss et al., 1983, *supra*; A.A. Weiss and E.L. Hewlett, *Ann. Rev. Microbiol.* 40:661-686, 1986) and antibodies to PT confer protection against the infection (H. Sato et al., *Infect. Immun.* 59:3832-3835, 1991; S.A. Halperin et al., *J. Infect. Dis.* 163:355-366, 1991).

To date, the cloning and expression of the PT S1 subunit in bacteria in an effort to create a protective vaccine has not resulted in production of a protective response. Vaccines produced by expression of S1 in *Escherichia coli* and in vaccine strains of *Salmonella typhimunium* (T. Dalla-Pozza et al., *Vaccine* 16:522-529, 1998; M.J. Walker et al., *Infect. Immun.* 60:4260-4268, 1992) failed to produce protective antibodies in the anti-recombinant S1 antisera, or such antibodies were present at levels too low to provide protection. Similar attempts using gram positive bacteria, such as *Bacillus subtilis* (K. Runeberg-Nyman et al., *Microb. Pathog.* 3:461-468,

1987, P. Saris et al., *FEMS Microbiol. Lett.* 56:143-148, 1990) and *Streptomyces lividans* (F.W. Paradis et al., *Appl. Microbiol. Biotechnol.* 45:646-651, 1996) resulted in expression of the S1 subunit as a soluble extracellular protein. Soluble antigens are more likely than particulate antigens to induce oral tolerance (the inability of an antigen to produce a specific antibody response due to a prior oral ingestion of the antigen) (S.F. Challacombe and T.B. Tomasi, in J. Brostoff and S. J. Challacombe (eds.), *Food Allergy and Intolerance*, Baillere Tindal, London Britain, 1987).

The insufficiency of such vaccines is explained by reference to the physiology of the respiratory and oral mechanisms of infection. In general, when microorganisms enter the respiratory tract, most of them are trapped by the mucus and are pushed upward to the back of the throat by the action of the cilia. They are subsequently swallowed and killed by the stomach acids (nonspecific defense). Oral pathogens, such as *B. pertussis,* can circumvent this defense by binding to ciliated cells, for example via adhesins such as FHA. The attachment may be abrogated by the action of antigen-specific secretory IgA (SIgA) antibodies present in the mucus by a process termed immune exclusion. The binding of these antibodies to *B. pertussis* effectively blocks the surface adhesins from mediating attachment and the bacterium will be rapidly cleared by the nonspecific defense. In the absence of specific SIgA antibodies, the bacterium will start the colonization process, causing localized inflammation. In response to inflammatory signals, serum components such as IgG and complement exude through the tight junctions between the epithelial cells, as well as between endothelial cells from the underlying blood vessels. If the epithelial surface is damaged by toxins produced by the pathogen, immune cells, such as macrophages and neutrophils, will be recruited to the area to mount a cellular attack on the pathogen.

Vaccination via intramuscular injections has not proven entirely successful in providing protection against microorganisms that enter the body via respiratory/oral mechanisms. Intramuscular vaccination typically elicits a systemic immune response which gives an elevated serum IgG response and cell-mediated response. Findings from Kingston Mills' group indicate that protective immunity conferred by whole-cell

vaccines, like immunity acquired from natural infection, is mediated by a Th1 (cellular) response (E.T. Ryan et al., *Immunol.* 93:1-10, 1998). However, B cell responses with antibodies to *B. pertussis* are also required for protection by intramuscular injection. (B.P. Mahon et al., *J. Exp. Med.* 186:1843-1851, 1997). In contrast, protective immunity conferred by acellular vaccines involves a more heterogeneous Th0 type of response (E.T. Ryan et al., 1998, *supra*; E.T. Ryan et al., 1997, *supra*).

Intraperitoneally injected heat-killed recombinant bacterial vaccines have been more successful. Mice injected intraperitoneally with the heat-killed recombinant *S. gordonii* showed excellent immunity to PT and histamine challenges. In this procedure, the gene (*spaP*) coding for the major surface protein antigen P1 was cloned from *S. mutans* NG5 (S.F. Lee et al., *Infect. Immun.* 56:2114-2119, 1988) and DNA coding for the N-terminal 179 amino acid of the S1 subunit was cloned into *spaP* creating a SpaP-S1 fusion (S.F. Lee et al., *Infect. Immun.* 67:1511-1516, 1999). The fusion gene was expressed in *S. gordonii* DL-1 and the gene product was found on the cell surface of this bacterium as shown by immuno-electron microscopy. The fusion protein was recognized by a monospecific polyclonal anti-PT antibody and the rabbit anti-fusion protein antibody recognized the native PT, indicating correct fusion had been made. The anti-fusion protein antibody showed some PT neutralization activity in the Chinese Hamster Ovary (CHO) cell clustering assay.

Despite the successes of the art in designing vaccines against such pathogens for intramuscular or intraperitoneal delivery, there is a need in the art for compositions that can be delivered orally and/or intranasally to stimulate protection against infection by a pathogen, such as a mucosal pathogen.

SUMMARY OF THE INVENTION

In accordance with the present invention, there are provided compositions for the stimulation of protection against infection by at least one pathogen. Invention compositions comprise a live commensal oral organism genetically modified so as to express at least one immunogenic fragment of said pathogen. In alternative

embodiments, the oral commensal organism is genetically modified to express a plurality of immunogenic fragments, for example a plurality of the immunogenic fragments associated with the surface of the organism. The commensal oral organisms presently preferred for use in the invention compositions are live *Streptococcus* that has been genetically modified to express one or more immunogenic fragments derived from a mucosal pathogen, such as the pertussis toxin of *Bordetella pertussis*, optionally further expressing a mucosal adjuvant such as cholerae toxin subunit B.

In accordance with another embodiment of the present invention, there are provided methods for prophylactically treating a host against infection by a pathogen. Invention treatment methods comprise orally or intranasally administering to the host an effective amount of an invention composition comprising a live oral commensal organism as described herein. In response to the invention treatment method, the host mounts a mucosal immune response to the immunogenic fragment of the pathogen by generating secretory IgA (sIgA) antibodies against epitopes contained in the immunogenic fragment(s). In many cases, serum antibodies against epitopes contained in the immunogenic fragment(s) are also developed in the host. In a preferred embodiment, the immunogenic fragment(s) are derived from one or more surface adhesins of a mucosal pathogen so that sIgA antibodies generated by the host immune response system will bind to and neutralize the invading pathogens, for example, by blocking the surface adhesins of invading pathogens from mediating attachment to mucosal surfaces. This allows the invading bacteria to be rapidly cleared by nonspecific defense mechanisms of the host.

In accordance with yet another embodiment of the present invention, there are provided methods for chronic immunization of a host against infection by a pathogen. Invention chronic immunization methods comprise orally or intranasally administering to the host an effective amount of an invention composition comprising a live oral commensal organism as described herein. The genetically modified live oral commensal organism according to the invention provides chronic immunization by persisting in the oral environment of the host and continuously exposing (and

reexposing) the host to the immunogenic fragment, thereby administering long-term immunogenic stimulation against the pathogen and consequent long-term protection to the host.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B collectively provide a schematic drawing of the preparation of a plasmid useful for obtaining a live oral commensal organism genetically modified to express an immunogenic fragment of the S1 subunit of pertussis toxin (PT).

Fig. 2 is a schematic drawing showing the preparation of a suicide plasmid pSL1 (pRJM4) from pRJMIII.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the present invention there are provided compositions for the stimulation of protection against infection by at least one pathogen. Invention compositions comprise a live commensal oral organism genetically modified so as to express at least one immunogenic fragment of said pathogen. Invention compositions have utility as an oral vaccine, thus, the immunogenic fragment(s) contained therein is (are) non-toxic to a host receiving the composition as well as to the oral commensal organism that is used as the carrier of the immunogenic fragment.

As used herein, the term "commensal oral organism" means that the native organism, once acquired in infancy, persists in a mammalian host in the oral cavity, (e.g., on the oral-pharyngeal mucosa, the dorsum of the tongue, and dental surfaces) under normal conditions (i.e., absent extensive antibiotic treatment to which the organism is sensitive) throughout childhood and into adulthood. Optionally, the organism may also persist in other mucosal environments in the host, such as the vagina. The organism is not cleared from the host by the host's immune response to the organism. Thus, such a commensal oral organism is one for which the existence of natural antibodies to the native organism (i.e., unmodified in accordance with the invention) does not prevent the continued existence of the organism in the oral cavity.

The invention modified live commensal oral organism is designed to similarly persist in the oral cavity of the host despite the production by the host of antibodies against the organism itself or against the immunogenic fragment(s) expressed by the modified organism.

As used herein, the term "mucosal pathogen" means a pathogen capable in the natural state of invading, colonizing, and/or infecting a host via a mucosal surface of the oral/respiratory system of the host.

As used herein, the term "stimulation of protection against infection" means that the immune system of the host is stimulated sufficiently by the presence of non-toxic immunogenic fragment(s) of the pathogen(s) contained in the invention composition to generate antibodies, especially secretory IgA (SIgA), against the pathogen(s). In certain cases, the host may generate other types of immune response as well, such as IgM or IgG, against the immunogenic fragment(s) of the mucosal pathogen. So long as the invention genetically modified live oral commensal organism colonizes and persists in the oral cavity of the host, stimulation of the host's immune response is ongoing, for example, throughout childhood, puberty and adulthood. Consequently, once the host is inoculated with the modified organism, the need for administration to the host of "booster" doses of the invention composition is minimized or avoided so long as the organism persists.

Examples of oral commensal organisms useful in the practice of the present invention include members of the *Streptococcus*, *Neisseria*, *Haemophilus*, and *Lactobacillus* families (C. Russell and T.H. Melville, *J. Appl. Bactriol.*, 44:183-181, 1987; J. Carlsson, et al., *Caries Res.* 2:333-339, 1975; J. Carlsson et al., *J. Dent. Res.* 49:415-418, 1970). Among these bacteria, species of the viridans streptococci (in particular *S. salivarius* and *S. mitis*) will colonize the oral mucosa by about 2 months after birth, with *S. gordonii* and other viridans streptococci beginning to colonize by about 6 months of age. In infants as well as adults, these streptococci constitute the bulk of the cultivable bacteria on the oral-pharyngeal mucosa, the dorsum of the tongue, and in dental plaque. The presence of viridans streptococci as commensal

organisms is important to the well-being of the host because they play a significant role in the survival of a neonate by excluding pathogens (P.A. Mackowiak, *N. Engl. J. Med.* 307:83-86, 1982). Hence such organisms as *Streptococcus gordonii, Streptococcus salivarius, Streptococcus mitis*, and the like, are presently preferred for use as the carrier organism in the practice of the present invention.

The native oral commensal organism is itself capable of eliciting at least a secretory immune response in a host. For example, viridans streptococci are capable of eliciting immune responses in infants and children (D.J. Smith and M. A. Taubman, *Critical Rev. Oral Biol. Med.* 3:109-133, 1992). SIgA with specificity for *S. mitis* and *S. salivarius* cells and their extracellular antigens can be detected in saliva of infants as young as 5 weeks of age (D.J. Smith et al., *Oral Micro. Biol. Immunol.* 5:57-62, 1990). This antibody response continues to increase in intensity and specificity during the first few years of life (D.J. Smith and M.A. Taubman, 1992, *supra*; D.J. Smith et al., 1990, *supra*) and probably reaches a maximal level in the later part of childhood. In adults, SIgA against viridans streptococci are consistently found in saliva, tears and colostrum (R.R. Arnold et al., *Infect. Immun.* 14:355-362, 1976; O.-P.J. Lehtonen et al., *Infect. Immun.* 43:308-313, 1984; M.R. Allansmith, et al., *Infect. Human* 35:202-205, 1982). Studies by J. Mestecky et al. (*J. Clin. Invest.* 61:731-737 1978) and J.R. McGhee et al. (*Adv. Exp. Med. Biol.* 107:177-184, 1978) of orally ingested *S. mutans* in humans have demonstrated the simultaneous appearance of anti-*S. mutans* SIgA antibodies in saliva and tears. The most probable explanation for such a response is the stimulation of the gut-associated lymphoid tissue (i.e. Peyer's patches) cells subsequent to the swallowing of bacteria or their antigenic products, resulting in the homing of sensitized B-lymphocytes to salivary and other secretory glands. These B-lymphocytes later differentiate into IgA-producing cells at these glands. In addition to secretory immune response, serum antibodies (IgG and IgM) to viridans streptococci are also present naturally in children (D.J. Smith et al., 1990 *supra*; Z. Luo et al., *J. Dent. Res.* 67:554-560, 1988) and adults (D.J. Smith et al., 1990 *supra*; O.-P.J. Lehtonen et al., 1984, *supra*; M.R. Allansmith, et al., 1982, *supra*).

Clearly, the presence of these natural antibodies does not prevent the continued existence of viridans streptococci in the oral cavity, as mammals, including humans, continue to harbor these bacteria after acquiring them during infancy. These organisms may persist because the level of antibodies present is relatively low. Alternatively, other mechanisms, such as production of IgA proteases by some of these bacteria (M. Kilian et al., *Microbiol. Rev.* 52:296-303, 1988) or active shedding of bound antibodies (S.F. Lee, *Infect. Immun.* 63:1940-1946, 1995) may be the reason. Whatever the mechanism for the persistence of these pathogens in mucosal tissue of mammals, the ability of resident oral streptococci like *S. gordonii* to elicit immune responses, especially a mucosal response, is exploited in the present invention for the prevention of diseases caused by infectious agents that normally do not persist in or colonize the oropharynx.

S. gordonii is commonly found on the human oral mucosa as well as in dental plaque. Because of its ability to elicit immune responses in infants and children, *S. gordonii* is particularly suitable for use as the oral commensal pathogen vehicle in the practice of the invention, e.g., for use to prevent diseases in children. The persistent nature of *S. gordonii* in the oral cavity indicates that after an initial inoculation of an infant or child with the invention live oral vaccine, the modified pathogen will colonize, replicate, and persist on the oral mucosa. Therefore, subsequent or repeated inoculation is minimized or not required. This characteristic, together with the ability to induce immune responses as long as the modified pathogen is present in the oral cavity, make the invention compositions a potentially economical vaccine system. In preferred embodiments, only one oral administration, preferably soon after birth, provides protection to the mammalian host for life.

Furthermore, as oral streptococci are known to be transmitted from mother to child, presumably through saliva (J. Carlsson et al., 1970, *supra*; D.J. Smith et al., 1990, *supra*), immunized mothers may transmit the vaccine strain to their babies. It is believed that oral commensal organisms are spread between adults by a similar mechanism. This natural means of inoculation using invention compositions and

methods may provide a solution to the lack of vaccination infrastructure in the developing world.

Other advantages of using the invention composition as a live oral vaccine include its simplicity of administration and potential for eliminating the local discomfort (redness and swelling) and systemic manifestations (fever, headache, and malaise) that commonly result from conventional vaccination.

In one embodiment, the oral commensal organism in the invention composition is modified to express one or more non-toxic immunogenic fragments of a single pathogen. If the pathogen is *Bordetella pertussis,* the preferred immunogenic fragment is derived from the pertussis toxin, especially the N-terminal 179 amino acids of the S1 subunit of the pertussis toxin (PT). PT is an AB toxin with A-promoter (S1 subunit) being the toxic subunit and B-oligomer being the pentamer that binds to cell receptors and translocates S1 subunit across the cell membrane. The S1 subunit is composed of 234 amino acids and is immunodominant. S1 contains 3 or 4 T-cell epitopes (M.T. De Magistris, et al., *J. Exp. Med.* 169:1519-1532, 1989; J.R. Oksenberg, et al., *J. Immunol.* 143:4227-4231, 1989; K.J. Kim, et al., *J. Immunol.* 144:3529-3534, 1990) and 5 B-cell epitopes (P. Askelof, et al., *J. Infect. Dis.* 157:738-742, 1988). Antibodies against the S1 subunit have been shown to neutralize PT *in vitro* and to protect mice from aerosol *B. pertussis* infection (H. Sato, et al. 1991, *supra*, S. A. Halperin et al., 1991, *supra*).

The B oligomer is composed of 1 subunit each of S2, S3, and S5 and two subunits of S4. S2 and S3 mediate adherence of the toxin to host cells. Although S2 and S3 have >80% amino acid sequence identity, they bind to different receptors on host cells. S2 binds specifically to lactosylceramide, a glycolipid found primarily on ciliated respiratory cells. S3 binds to a ganglioside found on phagocytic cells. Similar to S1 subunit, antibodies against B oligomer or S2 and S3 subunit have been shown to confer protection against *B. pertussis* infection in animal models, but are less effective than antibodies to S1 (S. A. Halperin et al., 1991, *supra*).

In some cases, as needed, the host's immune response to native invasion by a single pathogen can be enhanced by modifying the oral commensal organism to express a multiplicity of different non-toxic immunogenic fragments derived from a single species of pathogen. For example, the oral commensal organism can be modified to express a combination of immunogenic fragments from *Bordetella pertussis*, such as, for example, fragments selected from the pertussis toxin, filamentous hemagglutinin, pertactin and fimbriae 2 and 3 proteins, and the like.

In accordance with another embodiment of the invention, the oral commensal organism is modified to express a plurality of immunogenic fragments derived from more than one pathogen that has an oral/respiratory mechanism of colonization prior to infection and disease. Exemplary pathogens from which the immunogenic fragments can be derived include *Bordetella pertussis*, Respiratory Syncytial Virus (RSV), poliovirus, *Mycoplasma pneumoniae*, meningococcus, pneumococcus, rotavirus, influenza virus, influenza bacteria (e.g. *Haemophilus influenzae* type b), parainfluenza, and the like, and combinations of two or more thereof.

In some cases, immunogenic fragment(s) of a pathogen included in invention composition(s) are sufficient to generate a serum antibody response against the corresponding pathogen as well as a mucosal response, such that the serum antibodies developed in the host prevent the binding of the corresponding organism. In such cases, it is not necessary that the pathogen whose immunogenic fragment(s) are used in the invention composition be a mucosal pathogen. Examples of such non-mucosal pathogens against which protection can be afforded to the host by use of the invention compositions and methods include *Corynebacterium diphtheriae, Neisseria gonorrhoeae, non-typeable Haemophilus influenzae, Chlamydia pneumoniae, Chlamydia trachomatis, Moraxella catarrhalis*, and the like. Non mucosal pathogens against which protection can be afforded to the host by use of the invention compositions and methods include hepatitis B virus, *Clostridium tetani*, and the like.

In accordance with yet another embodiment of the present invention, the oral commensal organism is modified to express both immunogenic fragment(s) as

described herein, and at least one mucosal adjuvant, e.g., cholerae toxin subunit B (CtxB), ADP- ribosylating exotoxin, E. coli heat-labile enterotoxin, Clostridium difficile toxin, and the like.

Cholera toxin (CT) is an AB toxin produced by the bacterium *Vibrio cholerae*. The A subunit is the toxic component of the toxin, while the B subunit, a pentamer, is responsible for binding the toxin to the GM1 ganglioside on host cells. CtxB is a well documented mucosal adjuvant. CtxB has been used as a mucosal antigen in several animal models and is also a constituent of the oral cholera vaccine for human use (J. Holmgren, nature 292:413-417, 1981; J. Holmgren et al., Am. J. Trop. Med. Hyg. 50 (suppl.):42-54, 1994). CtxB has also been employed as a mucosal carrier for protein and polysaccharide antigens. When the CtxB-protein or polysaccharide antigens were given intranasally, perorally, or intravaginally, mucosal immune responses to the antigens have been observed (G. Hajishengallis et al, J. immunol. 154:4322-4332, 1995; C. Berquist et al., Infect. Immun. 63:2021-2025, 1995; M.W. Russell, et al., Infect. immun. 64:1272-1283, 1996).

CtxB has been expressed in *Lactobacillus* (P. Slos et al., FEMS Microbiol Lett 69:29-36, 1998) and *Bacillus brevis* (Y. Ichikawa et al., FEMS Microbiol. Lett. 111-219-224, 1993). These studies were aimed at large scale production of CtxB for easy purification to be used as an oral protein vaccine. Harokopakis et al (Infect. Immun. 65:1445-1454, 1997) has described the expression of CtxB in a *Salmonella typhimurium* vaccine strain expressing a saliva binding region (SBR) of a streptococcal protein. Immune responses to SBR were noted following inoculation of mice with the recombinant *S. typhimurium*.

In a specific embodiment of the present invention, the CtxB is produced by *S. gordonii* expressing pertussis vaccine antigens. CtxB is secreted by *S. gordonii* and forms a chimeric structure with the pertussis antigen fused to the A2 moiety of CT. In the native CT molecule, A2 forms noncovalent linkages between the toxic A1 subunit and the B subunit. Hence, the pertussis antigens-A2-CtxB chimeric structure mimics

the native CT molecule except the toxic A1 subunit is replaced by the pertussis antigens.

Thus, in accordance with one aspect of the invention, the gene coding for CtxB can be cloned behind the *S. mutans* SpaP promoter and signal sequence and introduced into the chromosome of *S. gordonii* DL-1. The DNA coding for the A2 moiety of CT can be cloned into the various constructs (e.g. SpaP-S1, SpaP-S1S3FHA) creating an in frame fusion. The new fusion gene (e.g. SpaP-S1-A2) is then introduced into the CtxB-producing *S. gordonii*. The recombinant *S. gordonii* produces and secretes SpaP-S1-A2 protein and CtxB protein outside the cell. Once, they are outside the bacterial cell, SpaP-S1-A2 forms a chimeric structure with CtxB. Because of the high affinity of CtxB for GM1 ganglioside, the chimeric structure specifically binds to M cells (GM1 ganglioside producing cells) in the mucosal lymphoid tissues and delivers the SpaP-S1 antigen to the immune cells. Hence, following oral colonization and maintenance of the live recombinant *S. gordonii* , the chimeric structure will be continuously produced to elicit a long-lasting mucosal immune response to the antigens and CtxB.

To modify the organism according to the invention to express a plurality of immunogenic fragments derived from more than one pathogen, it is presently preferred to insert nucleic acid encoding the multiplicity of immunogenic fragments as a fusion protein in a surface protein of the invention organism. For example, *S. gordonii* produces a surface multi-functional adhesin (CshA) of 259 kDa (R. McNab et al., *Mol. Microbiol.* 14:743-754, 1994) and can surface-express the 187 kDa SpaP-S1 fusion protein.

Accordingly, as illustrated in Example 1 below, *S. gordonii* can be modified to express surface fusion proteins having a polyvalent antigenic insert in the size range of about 187 kDa with assurance that the protein will be correctly processed and surface-localized in this bacterium. However, if the immunogenic fragments within the fusion protein do not properly fold so as to retain their immunogenicity (as

determined by competitive ELISA experiments), it is recommended that the number of antigens fused together be reduced so that the size of the antigenic insert is correspondingly reduced. Alternatively, each antigen can be expressed in a separately modified oral commensal organism, such as a separately modified *S. gordonii,* and the host can be inoculated with a mixture of the recombinant organisms, with each member of the mixture containing a transformant expressing a different immunogenic fragment. Such a mixture of recombinant organisms can be administered to the host by a variety of methods, for example, individually, spaced apart by any convenient time interval, in combinations of two or more thereof, or all at once.

Known assays and mouse models can be used to determine that a given modified live oral commensal organism is both non-toxic and capable of generating protective antibodies in a host. For example, a CHO cell assay can be used to determine the level of ADP-ribosylation activity of the S1 immunogenic fragment. The N-terminal 179 amino acid fragment of S1 subunit has been shown to be non-cytotoxic to CHO cells when fused to the diphtheria toxin A fragment and to require only 1% of the ADP-ribosylation activity catalyzed by the native S1 subunit of PT (J. T. Barbieri et al., *Infect. Immun.* 60:5071-5077, 1992). Example 2 herein describes an ADP-ribosylation assay for determining that the fusion protein containing the S1 subunit used in the invention composition retains minimal ADP-ribosylation activity. If such an assay determines that the fusion protein retains the ADP-ribosylation activity, site-directed mutagenesis may be used to mutate the arg and glu residues at positions #9 and #129 to lys and gly, respectively, to detoxify the fragment. M. Pizza et al. (*Science* 246:497-499, 1989) has shown that when these two residues were altered, PT became non-toxic. Those of skill in the art will know how to use similar assays or other types of assays to assure that the immunogenic fragment(s) inserted into the invention composition are not toxic either to the oral commensal organism that expresses the fragments or to the host to which it is administered in accordance with the invention.

Whatever the derivation of the fragments of pathogens, it is preferred that the immunogenic fragments are associated with the surface of the modified oral

commensal pathogen in invention composition(s). Thus, in the construction of live oral vaccines, it is desirable to develop genetic constructs that deliver the expressed foreign antigens to the cell surface where their antigenic determinants can be exposed to elicit immune responses. Being localized on the cell surface (cell-associated), the antigens are presented as particulate forms, which are more immunogenic than soluble (secreted) antigens. Particulate antigens also have the advantage over soluble antigens in that they are less likely to induce oral tolerance (the inability of an antigen to produce a specific antibody response due to a prior oral ingestion of the antigen) (S.F. Challacombe and T.B. Tomasi, in J. Brostoff and S. J. Challacombe (eds.), *Food Allergy and Intolerance*, Baillere Tindal, London Britain, 1987). The resistance to tolerance of the host is a characteristic particularly desirable in the invention compositions for persistence of the carrier pathogen in the oral cavity of the host, and hence, for long-term protective effect without administration of booster immunizations. In addition, the resistance to tolerance of the host is required to extend protection in the host beyond infancy and childhood into puberty and adulthood.

Therefore, it is preferred to modify a gene coding for a major surface protein antigen or a surface adhesin to express the immunogenic fragment as a fusion protein. For example, SpaP can be surface-expressed in *Streptococcus*, such as *S. gordonii* (M.K. Homonylo-McGavin and S.F. Lee, *J. Bacteriol.* 178:801-807, 1996; S.F. Lee et al., 1999, *supra*). When the pathogen is a *Streptococcus*, the gene (*spaP*) coding for the major surface protein antigen P1 can be modified so as to encode an in-frame fusion between SpaP and the immunogenic fragment. In addition to its role as a carrier of the immunogenic fragment in the invention composition, SpaP itself is highly immunogenic (N. Toida et al., *Infect. Immun.* 65:909-915, 1997; G. Halishemgallis et al., *J. Immunol.* 154:4322-4332, 1995); hence it serves as an excellent adjuvant for the immunogenic fragment of a mucosal pathogen in the invention composition. Finally, SpaP, like other surface proteins (e.g. protein A of *Staphylococcus aureus* (H. Ton-That et al., *J. Biol. Chem.* 272:22285-22292, 1997)), is covalently linked to the peptidoglycan (M. K. Homonylo-McGavin, *Thesis,*

University of Manitoba, 1996) via its 6 amino acid C-terminal motif. This linkage provides a natural peptidoglycan adjuvant to SpaP and the immunogenic fragment of a mucosal pathogen in the invention composition.

Sequence analysis of the spaP gene reveals that SpaP has a highly organized molecular structure (C. Kelly et al., *FEBS Letts* 258:127-132, 1989). The protein contains a four-part structure: (a) a 38-amino acid leader peptide at the N-terminus, which is cleaved during secretion, (b) an alanine-rich region comprising three 82-residue tandem repeats, (c) a proline-rich region comprising three 39-residue tandem repeats, (d) and a C-terminal surface-localization domain. The C-domain shares similar characteristics with other gram-positive surface proteins and is responsible for surface localization of antigen P1 in *S. mutans*, as well as in *S. gordonii* and *Enterococcus faecalis* (M.K. Homonylo-McGavin and Lee, 1996, *supra*). Therefore, it is preferred to transform *Streptococcus gordonii* with a vector containing DNA encoding immunogenic fragment(s) within the DNA encoding the surface protein antigen P1 of *Streptococcus mutans.* A shuttle vector suitable for use in Streptococci is the *Escherichia coli-Streptococcus* shuttle vector pDL276 (G.M. Dunny et al., *Appl. Environ. Microbiol.* 57:1194-1201, 1991), which can be introduced into *S. gordonni* by natural transformation. Example 1 describes modification of spaP from *S. mutans* NG5 to contain a nucleic acid segment that encodes a 187 kDa SpaP-S1 fusion protein (S.F. Lee et al., 1988).

To prevent the loss of the shuttle plasmid during oral colonization in the absence of an antibiotic for which the shuttle plasmid contains a selection gene, the fusion gene can be delivered into the chromosome of the oral commensal organism via homologous recombination by transforming a suicide plasmid carrying a DNA fragment originated from the chromosome of the commensal organism. Example 2 herein describes use of a HppG knock-out mutant of *S. gordonii* that can colonize the oral cavity of mice as well as the wild-type for this purpose. Through homologous recombination, a plasmid containing both the DNA encoding the fusion protein and the suicide plasmid integrates into the chromosome of the commensal organism. The

resulting transformants maintain the fusion gene indefinitely as it becomes part of the bacterial genome.

Nucleic acid sequences encoding immunogenic proteins from pathogens, for example the adhesins used by the pathogen to adhere to and colonize mucosal surfaces, are readily available from public data bases, such as GenBank. Antibodies that bind to epitopes on such proteins are well known, can be generated using well known procedures, and are also available from such public repositories as the ATCC. Therefore, in selecting the immunogenic fragment(s) for insertion into a gene encoding the surface protein of the selected oral commensal organism, those of skill in the art can readily utilize a computer analysis of the crystal structure of the intact immunogenic protein, for example PT, to determine those fragments with the highest likelihood of folding to retain immunogenic structure (i.e., epitopes to which antibodies specific for the fragment bind) as is known in the art (P.E. Stein et al., *Structure* 15:45-47, 1994, B. Hazes et al., *J. Mol. Biol.* 258:661-671, 1996).

For detection of toxicity of the PT S1 fragment, additional assays, such as the ADP- ribosylation (ADPR) assay, are also performed to assure that the modified organism is not toxic. For the anti-ADPR activity, the method used by H. Sato et al. (1991, *supra*) can be used. Briefly, CHO cells are lysed in a reaction buffer containing EDTA and cell membranes are collected by centrifugation. Native and antibody-reacted PT are incubated with the CHO cell membranes in a reaction buffer containing [^{32}P]NAD. The incorporation of [^{32}P]ADP into the transducin present in the cell membrane is measured by liquid scintillation counting. The [^{32}P]ADP ribosylated transducin is visualized by exposing the gel on an X-ray film.

The immunogenicity and protective effect of the invention compositions as oral vaccines can be tested in available mouse infection models, such as that for pertussis, which is illustrated herein in Example 2 below. For example, sera, saliva, and/or bronchoalveolar lavage fluids can be obtained from hosts immunized with the invention genetically modified oral commensal organisms and the samples can be analyzed to determine titers and specificity against the immunogenic fragment by

ELISA and Western blotting, respectively, using known methods and as illustrated in the Examples herein.

There is no pertussis animal model that adequately reflects all of the pathophysiology observed in infections caused by *B. pertussis,* for which humans are the only natural host. However, the mouse model (aerosol or intranasal inoculation) serves as an excellent model for pertussis disease that is often observed in young infants. The mouse model of pertussis has been established using aerosol infection and the histopathology characterized (S.A. Halperin et al., *Clin Invest* Med 11:297-303, 1988). Monoclonal antibodies against *B. pertussis* virulence factors, such as pertussis toxin and filamentous hemagglutinin are also known (S.A. Halperin et al., 1991, *supra*). Using these monoclonal antibodies and the mouse model of infection, the immune response to infection with pertussis and the protective effect of antibodies to pertussis has been established using an infectious aerosol challenge after immunization with one or more antigens. (S.A. Halperin et al., *Sixth International Symposium on Pertussis,* Bethesda, MD, 1990; S. A. Halperin et al., *Canadian Society of Clin Micro and Inf Dis.,* Halifax, NS 1990; S.A. Halperin et al., 1991, *supra*; S. Cockle et al., *33rd ICCAAC,* New Orleans, LA 1993). In young mice, the diffuse pulmonary infiltrates caused by a primary pertussis pneumonia, the lymphocytosis, and the hyperinsulinemia are mimicked closely. In older mice, non-lethal infections are induced which have many of the characteristics of pertussis in older infants; however, the mice do not display the clinical manifestations of the disease, particularly the paroxysmal cough characteristic of these infections. The rat model of pertussis described by D.E. Woods et al., *Infect Immun* 57:1018-1024, 1989 and further characterized by E. Hall et al., *J. Med. Microbiol.* 40:205-213, 1994 does produce paroxysmal coughing and other characteristic physiological changes; however, there is substantially less experience with this model in evaluating new candidate pertussis vaccines.

Similar animal models are known for other mucosal pathogens or can readily be developed by those of skill in the art. For example, an animal model for *Legionella pneumophilia* is known in guinea pigs (R.F. Berendt et al., *J. Infect. Dis.*

141:186-192, 1980), an animal model for *Mycoplasma pneumonia* is known in hamsters (J.V. Jemski et al., *Infection and Immunity* 16:93-98, 1977, an animal model for pneumococcus is known in mice (E. Rosenow et al., *Mol. Microbiol.* 25 (5):819-29, 1997), an animal model for meningococcus is known in mice (I.E. Salit and L. Tomalty, *Clin. Invest. Med.* 9 (20):119-23, 1986), an animal model for *Haemophilus influenzae* is known in rats (F.J. Wallace et al., *Am. Rev. Respir. Dis.* 140 (2):311-16, 1989, and the like.

In accordance with another embodiment of the invention, there are provided methods for prophylactically treating a host against infection by a pathogen. Invention treatment methods comprise orally or intranasally administering to the host an effective amount of an invention composition comprising a live oral commensal organism as described herein. The modified oral commensal organism used in accordance with the present invention is live when administered and naturally colonizes in the oral mucosa of the host. As used herein, the term "live" means that the invention modified organism is capable of propagation at 37° C. in a suitable culture medium, such as an aqueous suspension containing at least $2X10^3$ of said viable organisms per milliliter.

Preferably the host is administered the modified organism of the invention during infancy, but the host can be administered the modified organism at any age so long as a sufficient amount of the modified organism is administered to crowd out native (i.e., unmodified) species which may already be present in the host oral cavity. In human hosts, therefore, the modified organism is generally administered to an infant at birth up to about 6 months of age.

Administration of invention composition(s) orally or intranasally elicits in the host at least a mucosal immune response so that the disease process of the mucosal pathogen is stopped at the first step, i.e., adherence and colonization of the mucosal surface in the oral cavity of the host. Oral and nasal immunization preferentially elicits a Th2 response that directly correlates to an elevated level of antigen-specific SIgA antibodies. It has been shown previously that nasal immunization of mice with

B. pertussis whole cells or its antigens induces an elevated SIgA to the antigens in saliva and bronchoalveolar lavage fluids (A.K. Berstad et al., *Vaccine* 15:1473-1478, 1997; M. Roberts et al., *Vaccine* 11:866-872, 1993; Lipscombe et al., *Mol. Microbiol.* 5:1385-1392, 1991). However, the response to such whole cell immunizations is short-lived, due to the lack of memory of mucosal immune cells. The present invention provides a means for overcoming such shortfalls. In accordance with the present invention, the mucosal immune response of the host is long-term because the antigens required to continuously provide the stimulation necessary to maintain the mucosal immune response are continuously expressed by the live modified organism colonizing the oral cavity of the host so that subsequent or repeated inoculation is minimized or unnecessary once the colonization of the oral mucosa has been well established.

Other advantages of using the invention treatment methods as compared with other immunization methods include the elimination of local discomfort (e.g. the redness and swelling associated with injections) and the avoidance of systemic manifestations (e.g., fever, headache, and malaise) that can result from conventional vaccination.

Therefore, in accordance with the present invention there are further provided methods for chronic immunization of a host against infection by a pathogen. The invention chronic immunization method comprises orally or intranasally administering to the host an invention composition comprising a live oral commensal organism genetically modified as described herein. The invention chronic immunization method further comprises establishing sufficient colonies of the modified oral commensal organism in the host to continuously stimulate the mucosal immune system of the host against the target pathogen(s). The genetically modified live oral commensal organism provides chronic immunization because the organism persists in the oral environment and continuously exposes the host to the immunogenic fragment to administer long-term immunogenic stimulation against the pathogen and consequent protection to the host.

The genetically modified organism is administered in an "effective amount" in the invention methods. An effective amount is the quantity of the modified live oral commensal organism necessary to colonize the oral cavity of the host so as to prevent, or at least partially inhibit, infection by the pathogen(s) from which the immunogenic fragment expressed by the modified organism is derived.

A host is any mammal, preferably a human. The invention method and compositions can be used with a wide variety of domestic animals, as well as man. Included among domestic animals which are treated by the invention compositions or could be treated, if susceptible to infection by bacterial or viral pathogens as disclosed herein, are cows, pigs, horses, goats, rabbits and sheep, to name the more important domestic animals. For example, the methods and procedures disclosed herein can be used according to the invention to develop a composition useful for treatment of *B. bronchiseptica* (kennel cough) in dogs, pneumonia in cats, atrophic rhinitis and pneumonia in pigs, *B. avium* in turkeys, and the like.

Invention composition(s) may be administered in any of a variety of ways, e.g., any conventional methods for administering a live bacterium either orally or intranasally being applicable. These include oral administration, on a solid physiologically acceptable base, or in a physiologically acceptable dispersion that is non-toxic to the modified organism, or the like. The dosage of the invention composition (number of modified organisms, number of administrations) will depend on a number of factors, such as the route of administration and the weight and age of the host (e.g., whether already infected with a native strain of the unmodified organism) and will vary according to the species to be protected. For neonate mice, of weight 2.5 to about 3.5 g, administration of a single dose of about $3x10^5$ to about $3x10^9$ of live *S. gordonii* modified to express the SpaP-S1 fusion protein, either by pipeting 100 µl of a 24-h old culture into the oral cavity and/or nostrils, or by swabbing the oral cavity with a cotton swab saturated with the 24-h culture, with repeated inoculations at 24 and 36 hours later, appears both safe and effective.

The formulations employed for delivery of the live organisms may be varied widely, and may optionally include one or more immunological adjuvants to enhance immunogenic response of invention compositions, e.g., aluminum hydroxide, monophosphoryl lipid A, trehalose diesters, saponins, muramyl dipeptide, and the like, as well as mixtures of any two or more thereof. For oral administration, the live vaccine may be provided on a sugar or bread cube, in buffered saline, in a physiologically acceptable oil vehicle, or the like. For example, invention modified live organisms can also be administered orally on bread or sugar cubes moistened with 0.1 ml of broth culture provided after six hours deprivation of other food. Alternatively, a variety of formulations, such as chewable capsules, elixirs, syrups, wafers, and the like, can be used so long as the formulation is not toxic to the modified organisms and an effective amount of the immunogenic fragment(s) is contained therein.

For intranasal administration, invention organisms are preferably employed as an aqueous suspension, which can be readily administered to the host by application to the nasal mucosa. For example, the aqueous suspension of cells may be introduced into the nasal passages in a measured amount by means of a syringe, or a measured amount of the aqueous cell suspension may be sprayed into the nostrils. The cell suspension should therefore be sufficiently liquid so that it is readily administerable or sprayable. Depending on the species to which the invention composition is to be administered and the amount of the cell suspension to be intranasally administered, the concentration of viable cells may vary over a wide range. For example, concentrations of from about 10^3 up to about 10^8 cells can be used. Even with small animals such as guinea pigs and rabbits, however, it will usually be desirable to administer at least one thousand viable cells per nostril, making a total dose of $2x10^3$ cells. Larger doses for larger mammals, such as humans, are desirable, such as at least $1x10^5$ up to about $1x10^7$ viable cells per milliliter.

While a degree of immunization may be obtained by introducing invention composition into one nostril of the host, the preferred procedure is to introduce approximately equal amounts of the aqueous cell suspension into both nostrils (nares).

The dose volume tends to be limited by the amount of liquid that the nostrils can retain. For example, the total dose (both nostrils) may range from about 0.5 ml up to about 1.5 ml of the suspension. As indicated, this dose may be divided approximately equally between the two nostrils. For example, from about 0.25 ml to about 0.7 ml of the suspension may be introduced into each nostril. A convenient dose size is 0.5 ml per nostril.

For the purposes of the present invention, cell counts may be made by standard procedures. Cell concentrations per milliliter of invention composition are determined by plating the cells and counting the CFU (colony forming units). The propagation for determining CFU may be on standard plates, such as 5% horse blood agar, Todd-Hewitt agar or Brian Hearth infusion agar.

One or more additional administrations may, optionally, be given, as needed, to assure that the oral cavity of the host has been effectively colonized, usually at convenient intervals such as about three times in a week, or at about 12 and about 48 hours after the initial exposure. Once the modified live organisms have become established in the oral cavity of the host, further boosters are not required unless, for some reason, the established colonies are eradicated. In an alternative embodiment of the invention following the method of D. Medaglini et al., *Proc. Natl. Acad. Sci. USA* 92:6868-6872, 1995, if the modified organism is antibiotic-resistant due to its inclusion of an antibiotic resistance selection marker, such as kanamycin or spectinomycin, the host is administered the corresponding antibiotic, for example in drinking water, prior to administration of the invention modified organism to lower the load of oral microflora prior to oral or intranasal inoculation.

To determine whether the invention modified organism is successfully colonized in the oral cavity, microbiological samples, for example using cotton swabs can be obtained on day 4 and every 7 days after the oral inoculation for testing using known methods and as disclosed in the Examples herein.

The vaccines of this invention and the results which can be obtained by their use are further illustrated by the following experimental examples.

EXAMPLE 1

Construction of SpaP-S1 fusion protein.

An in-frame fusion between *S. mutans* SpaP (antigen P1) and the *B. pertussis* PT S1 subunit was constructed as follows. The S1 gene (coding for amino acid residues 2 to 233 of the mature S1) was amplified by PCR from the PT operon carried on pPTX42 (*E. coli* ATCC 67046 (C. Locht and J.M. Keith, *Science* 232:1258-1264, 1986) by standard methods using the primers:

5'-GATCCTCCCGCCACCGT-3' (SEQ ID NO:1) and

5'-GGATCGATAACGAATACGCGATGCT-3' (SEQ ID NO:2)

The underlined bases are added sequence for a *Cla*I site). The amplicon was treated with Klenow fragment, restricted with *Cla*I, purified from agarose gels with a Gene-Clean™ kit (Bio. 101, La Jolla, Calif.), and ligated into the *Eco*RV-*Cla*I sites of pN1C4, a pUC18 derivative carrying the 3' DNA of *spaP* from *S. mutans*, coding for the C-terminal 144 amino acids containing the surface protein anchoring domain of SpaP (M.K. Homonylo-McGavin and S.F. Lee, 1996, *supra*). The ligated DNA was transformed into competent *E. coli* HB101, and the resulting plasmid was designated pPTS1. To provide the fusion gene with the *spaP* promoter, the 1.5-kb *Sma*I-*Xba*I fragment from pPTS1 was cloned into the *Eco*RV-*Xba*I sites of pSMI/II, a pUC18 derivative carrying the complete *spa*P gene (Kelly et al., *supra*). The resulting plasmid isolated from one of the *E. coli* HB101 transformants was named pRJMI. The 5.6-kb *Kpn*I-*Sca*I fragment from pRJMI was further cloned into pDL276, creating pRJMII. pRJMIII was further constructed by ligating the 10.0-kb *Nru*I-*Kpn*I fragment from pRJMII to the 4.5-kb *Eco*RV-*Kpn*I fragment from pSMI/II.

In summary, the initial gene fusion was constructed on a pUC18-based plasmid to create pRJMI. To facilitate the expression in streptococci and to avoid the

use of the Amp^R marker, the fusion gene was cloned into pDL276, an *E. coli*-streptococcus shuttle vector (G.M. Dunny et al., 1991, *supra*), creating pRJMII. pRJMII was introduced into *S. gordonii* DL-1 by natural transformation (M.K. Homonylo-McGavin and S.F. Lee, 1996, *supra*). Transformants were selected on Todd-Hewitt agar containing 250 μg of kanamycin/ml. Several transformants were obtained. These transformants were treated with mutanolysin (M.K. Homonylo-McGavin and S.F. Lee, 1996, *supra*), followed by boiling with the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer of U.K. Laemmli, *Nature* 227:680-685, 1970. In Western blotting, proteins reacted with the anti-SpaP monoclonal antibody 4-10A (dilution, 1/7000 (Ayakawa et al., *Infect. Immun.* 55:2759-2767, 1987) were compared with proteins reacted with the *S. gordonii* -absorbed rabbit anti-PT antibodies (dilution, 1/100). Lane 1 contained recombinant SpaP-S1 *S. gordonii* RJMIII; lane 2 contained recombinant *S. gordonii* DL-1 SMI 11-3 expressing only SpaP; lane 3 contained parent *S. gordonii* DL-1.S proteins extracted from cells by boiling SDS-PAGE sample buffer (20 μl). The approximately 187-kDa SpaP-S1 fusion protein was revealed by the anti-PT antibodies. This immunoreactive protein matched the predicted size of SpaP-S1 carried on pRJMII. However, when the transformants were analyzed by whole (intact)-cell enzyme-linked immunosorbent assay (ELISA) (M.K. Homonylo-McGavin and S.F. Lee, 1996, *supra*) and immunoelectron microscopy, none of them showed an appreciable amount of the fusion protein on the cell surface.

Since *S. gordonii* DL-1 produces a number of high-molecular-weight (ca. 190- to 259-kDa) surface proteins (H.F. Jenkinson and R.J. Lamont, *Crit. Rev. Oral Biol. Med.* 8:175-200, 1997), the SpaP-S1 fusion protein expressed from pRJMII may be buried among these proteins. To further investigate this possibility, pRJMIII was further constructed by placing the S1 fragment close to the middle part of SpaP. In the construction, the unique *Nru*I site within the S1 sequence was used so that the final fusion protein contained only the first 179 amino acids of S1 inserted into the complete SpaP, creating a predicted mature protein of approximately 187 kDa. Initial immunoblotting of lysates of *E. coli* HB101 carrying pRJMIII indicated the reactivity

of an approximately 187-kDa protein band with the anti-PT antibodies, suggesting that the correct fusion had been made. Then pRJMIII was transformed into *S. gordonii* DL-1 and one of the transformants, *S. gordonii* RJMIII, was chosen for further studies.

The anti-PT antibodies used were generated by immunizing two New Zealand White rabbits with 0.5 ml of a whole-cell *B. pertussis* vaccine (Connaught Laboratories Ltd., North York, Ontario Canada) by subcutaneous injections, followed by booster injections with the same vaccine on days 20, 27, and 48 and a final booster with 110 μg of purified PT in Freund's incomplete adjuvant (Connaught Laboratories Ltd.) on day 65. The titers of the antisera against purified PT (20 ng/well; List Biological Laboratories, Inc., Campbell, CA.) were determined by an ELISA using methods described previously (S.F. Lee, 1995, *supra*). The titers of anti-PT antibodies were expressed as the reciprocals of the dilutions that produced A_{405} readings 0.05 higher than the preimmune sera. The titers were 12,800 and 6,400 for the sera from the two rabbits. To eliminate cross-reactivity, pooled sera were adsorbed with *S. gordonii* DL-1 cells before use. To achieve this, 1 liter of *S. gordonii* DL-1 grown in TYG (1% tryptone, 0.5% yeast extract, 0.3% K_2HPO_4, 0.2% glucose [wt/vol]) to the late-exponential phase of growth was centrifuged (at 10,000 X *g* for 15 min at 4°C). The cells were washed once with phosphate-buffered saline (PBS) and resuspended in 15 ml of PBS. Half of the cell suspension was incubated with 5 ml of the anti-PT rabbit sera at 4°C for 1 hour with occasional mixing. The suspension was centrifuged, and the remaining half of the cells was added to the supernatant and incubated for an additional hour. The suspension was again clarified by centrifugation, and the supernatant was used as absorbed anti-PT antibodies.

Expression and localization of SpaP-S1 fusion protein in *S. gordonii*.

When culture supernatant fluid and cell extracts of *S. gordonii* RJMIII were analyzed by immunoblotting, a strong, 187-kDa protein band and a weaker, 155-kDa band from the cell extracts were recognized by an anti-SpaP monoclonal antibody.

The same 187-kDa band from the cell extract was also recognized by the absorbed anti-PT antibodies. Samples obtained from *S. gordonii* DL-1 did not react with either of the antibodies. Samples prepared from *S. gordonii* DL-1/SMI/II-3 (M.K. Homonylo-McGavin and S.F. Lee, 1996, *supra*), a DL-1 transformant carrying the *spaP* gene, showed reaction with the anti-SpaP antibody, but not with the anti-PT antibodies. These results strongly indicate that *S. gordonii* RJMIII is expressing the correct SpaP-S1 fusion protein and that the fusion protein is mainly cell associated.

To provide evidence that the fusion protein is surface localized, *S. gordonii* RJMIII was analyzed by immunoelectron microscopy. Briefly, cells were harvested from 1.5 ml of a late-exponentially grown Todd-Hewitt culture, washed twice in PBS, resuspended, and incubated in 0.5 ml of 1% (wt/vol) gelatin in PBS at room temperature for 10 min. The cells were further treated with 20 mM glycine in PBS for 3 min and 1% (wt/vol) bovine serum albumin (BSA) in PBS for 2 min. After these blocking steps, the cells were resuspended in 100 µl of *S. gordonii*-absorbed anti-PT antibodies (dilution, 1/50) or a monoclonal antibody, 4-10A, to SpaP (dilution, 1/100) and were incubated with gentle rocking for 2 h. The cells were washed 4 times with PBS containing 0.1% BSA and were reacted with colloidal gold (diameter, 10mm)-conjugated goat anti-rabbit immunoglobulin G or goat anti-mouse immunoglobulin G (dilution, 1/20) for 1 h. The cells were then washed 4 times with PBS-0.1% BSA, followed by two washes with PBS, and were finally fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Cells were then sectioned and viewed under a Phillips 300 electron microscope. The fusion protein was clearly found on the cell surface after the cells had been reacted with the anti-SpaP monoclonal antibody or the anti-PT antibodies. In contrast, DL-1 cells treated the same way did not exhibit any immunogold particles. To further examine the location of the fusion protein expressed in *S. gordonii* RJMIII, cellular fractions were prepared from 1 liter of late-exponential culture grown in TYG. Proteins in the supernatant fluids were precipitated with $(NH_2)SO_4$, 70% (wt/vol) and dialyzed against 10 mM Tris buffer, pH 8 (final volume, 15 ml). Cells were washed and broken with a Mickle tissue disintegrator (M.K. Homonylo-McGavin and S.F. Lee, 1996, *supra*).

Cell walls were recovered by centrifugation (at 27,000 X *g* for 30 min at 4°C) and resuspended in 1 ml of 10mM potassium phosphate buffer, pH 7.5. The cell membrane was separated from the cytoplasm (12ml) by ultracentrifugation (at 50,000 x*g* for 1 h at 4°C) and resuspended in 0.25 ml of potassium phosphate buffer. Samples of 15, 1, 12, and 0.25 µl of the extracellular fraction (supernatant), cell wall, cytoplasm, and cell membrane, respectively, were analyzed by Western immunoblotting. These volumes represented proportional amounts of each of the subcellular fractions from the culture. SpaP-S1 was detected by the *S. gordonii*-absorbed rabbit anti-PT anti-bodies (dilution 1/200).

Results showed that the fusion protein was mainly associated with the cell wall with a small amount found in the extracellular fraction and trace amounts in the cell membrane and cytoplasmic fractions. These results strongly indicate that the fusion protein was cell surface localized in *S. gordonii* RJMIII, and they reconfirm the ability of the C terminus of *S. mutans* SpaP to anchor to the cell wall in *S. gordonii* (M.K. Homonylo-McGavin and S.F. Lee, 1996, *supra*).

Immunogenicity of the recombinant S1.

The immunogenicity of the SpaP-S1 fusion protein was investigated by immunizing New Zealand White rabbit with heat-killed (15 min at 100°C) *S. gordonii* RJMIII cells (10^{10} CFU in 1 ml of Freund's incomplete adjuvant) by using a protocol similar to that described above. The antibody obtained was found to react with the native PT in an ELISA although the titer of the anti-serum was relatively low (1,600).

In Western blotting, the anti-SpaP-S1 antiserum clearly recognized the S1 subunit of the native PT, suggesting that the recombinant S1 expressed by *S. gordonii* is capable of eliciting an immune response. Western blots showed the specific reactivity of the rabbit anti-SpaP-S1 serum with the S1 subunit of P1. The rabbit anti-PT antiserum (dilution, 1/200) reacted with the S1, S2 and S3 of PT(1 µg. List Biological Laboratories, Inc.). The rabbit anti-SpaP-S1 antiserum (dilution, 1/100) reacted with the S1 subunit of P1 only. Preimmune serum (dilution, 1/100) obtained from the same rabbit used to raise the anti-SpaP-S1 serum did not react with PT.

This finding is consistent with findings by others that recombinant S1 expressed in *E. coli, Salmonella,* and *B. subtilis* is immunogenic.

Protective effects of recombinant S1.

The ability of the rabbit anti-SpaP-S1 antiserum to neutralize the cytotoxic effect of PT was assessed by the Chinese hamster ovary (CHO) cell-clustering assay using the method described previously (S.A. Halperin, 1991, *supra*). In the assay, 4 times the minimum clustering dose of native PT (Massachusetts Biologics Laboratories, Jamaica Plain, MA.) was used to allow for a twofold variation. Neutralization titers of antibodies were expressed as the reciprocals of the dilutions which showed the complete neutralization of the cell-clustering effect of PT. The immune serum showed a weak neutralization titer of 4, while the preimmune serum did not show any activity (neutralization titer, 0). The anti-Spa-S1 antibody was subsequently concentrated sevenfold by $(NH)_2SO_4$ precipitation, (S.F. Lee, *Infect. Immun.* 60:4032-4039, 1992) and demonstrated a neutralization titer of 32. A rabbit anti-SpaP antibody prepared previously (S.F. Lee, 1992, *supra*) was similarly concentrated sevenfold and showed a neutralization titer of 2. These results suggest that the anti-SpaP-S1 antibody had a weak ability to neutralize the cytotoxic effect of PT on the CHO cells. Previous studies by J.T. Barbieri et al., *Infect. Immun.* 60:5071-5077, 1992 and P.H. Boucher et al., *Infect. Immun.* 62:449-456, 1994 also found that the anti-recombinant S1 antibodies showed weak neutralizing abilities in the CHO cell-clustering assay.

The *in vivo* protective effect of the recombinant S1 was assessed by the leukocytosis-promoting and histamine-sensitizing assays (S.A. Halperin, 1991, *supra*). A cohort of BALB c female mice (3 weeks old; $n = 5$) were immunized intraperitoneally (i.p.) with heat-killed (10 min at 100°C) *S. gordonii* RJMIII cells (10^9 CFU in 0.2 ml of Freund's complete adjuvant). The animals were boosted with freshly prepared heat-killed cells in Freund's incomplete adjuvant via the same route 2 and 3 weeks later. A cohort of non-immunized mice(n=6) were used as a control. At 7 days after the last booster, sera were obtained from the animals; the immunized

mice had a titer of 12,800 against the native PT by ELISA. Each animal from the immunized and control groups was then injected i.p. with 0.5 μg of native PT (Massachusetts Biologics Laboratories) in 0.2 ml of PBS. The total leukocyte (WBC) count was determined with a hemacytometer on 1 μl of blood taken from the tail vein before and after PT injection. Before PT challenge, the control and immunized mice had average WBC counts of 9.14×10^9/liter (range, 4.5×10^9 to 12.2×10^9) and 8.28×10^9/liter (range, 5.5×10^9 to 10.0×10^9), respectively. Three days after PT injection, the control mice had an average WBC count of 29.8×10^9/liter (range, 26.5×10^9 to 35.0×10^9), 3.3 times higher than that before PT challenge. The average WBC count of the immunized mice was 11.5×10^9/liter (range, 8.75×10^9 to 15.75×10^9) after PT injection, 1.4 times higher than that before the challenge. Each animal was further given 2 mg of histamine diphosphate (Sigma Chemical Co., St. Louis, Mo.) i.p. 4 days after PT challenge, and deaths within 24 h of histamine administration were recorded. All five of the immunized mice survived the treatment, while only one of the six control mice survived. These results strongly suggest that the recombinant S1 fragment expressed by *S. gordonii* can induce protective antibodies in vivo.

EXAMPLE 2

Oral colonization and immune response to recombinant *S. gordonii* expressing S1 fragment.

As demonstrated in Example 1 above, the recombinant S1 subunit fragment clearly can elicit protective immune response to PT in mice. In the present experiment, the effectiveness of the recombinant antigen is tested against *B. pertussis* infection.

Delivery of SpaP-S1 fusion gene into chromosome.

The SpaP-S1 fusion protein gene is carried on the *E. coli*-streptococci shuttle plasmid (pRJMIII (kan^r, S.F. Lee et al., 1999, *supra)*) which is maintained in *S. gordonii* by kanamycin selection. In order to prevent the loss of this plasmid in the absence of kanamycin during oral colonization, the fusion gene is delivered into the

chromosome of *S. gordonii* via homologous recombination by transforming a suicide plasmid carrying a DNA fragment originated from the chromosome of *S. gordonii*. The method is that used by S. Lee to knock-out *spaP* in *Streptococcus* (S.F. Lee et al., *Infect. Immun.* 57:3306-3313, 1989). A DNA fragment coding for the oligopeptide-binding lipoprotein gene *hppG* is used from *S. gordonii*. Since *hppG* is not an essential gene, the HppG knock-out mutant can colonize mice as well as the wild-type (H.F. Jenkinson et al., *In Genetics of streptococci, enterococci and lactobacilli,* Ferretti J.J. (ed) American Society for Microbioloy, Washington DC., 1995). The 400 bp hppG DNA fragment has been cloned in pBluescript to create the plasmid pHppG. The suicide plasmid, pSL1 (or pRJM4) was constructed as shown in Figure 2 herein.

Briefly, a 400 bp DNA fragment of hppG, amplified by polymerase chain reaction (PCR) and cloned in pBluescript is then subcloned into pRJMIII and the streptococcal pVA380-1 replication origin on pRJMIII was deleted to prevent the plasmid from replication in *S. gordonii*. This suicide plasmid pRJM4 or pSL1 retaining the kan^r gene for selection was then transformed into *S. gordonii*. Through homologous recombination the plasmid integrates into the chromosome. Integration of the pRJM4 plasmid into the chromosome is verified by Southern analysis (S.F. Lee et al., 1989, *supra*). The resulting transformants maintain the fusion gene indefinitely as it now becomes part of the bacterial genome. Expression of SpaP-S1 by the new recombinants is confirmed by western blotting. Surface-expression of SpaP-S1 is confirmed by immunogold electron microscopy (S.F. Lee et al., 1999, *supra*).

Oral colonization.

Newborn mice, which are practically bacteria-free or are minimally colonized by bacteria, are selected to have the recombinant *S. gordonii* colonized and established in the murine oral cavity. Timed-pregnant BALB/c mice close to giving birth (20 days into pregnancy) are closely watched. Immediately after the baby mice arrive, they are inoculated with the *S. gordonii* strain, either by pipeting 100 µl of a 24-h old culture into the oral cavity or by swabbing the oral cavity with a cotton swab saturated with the 24-h culture. The neonatal mice are then returned to the mother.

The inoculation process is repeated two more times, 24 and 36 hours later. The neonatal mice are kept with the mothers until they are ready to be weaned (3 weeks old) and subsequently caged in groups. The mice are fed with a sterile normal diet with water *ad libitum*.

To minimize the cannibalistic loss of babies due to "stress" and manipulations, the pregnant mice are handled with gloved hands a few days before the arrival of babies and a latex glove and a cotton swab is placed in the cage to allow the mothers to become familiar with the odor of latex and cotton. The bedding is changed before the arrival of the babies to ensure the animal has settled in.

Following the method of D. Medaglini et al., *Proc. Natl. Acad. Sci. USA* 92:6868-6872, 1995, since the recombinant *S. gordonii* is kanamycin-resistant, 3-week-old BALB/c mice have been successfully colonized with the pRJM4-modified *S. gordonni*. The mice were fed with 500 μg/ml kanamycin in drinking water for two days prior to inoculating the oral cavity and nares with 100 μl of an overnight culture of the pRJM4-modified bacteria to lower the load of oral microflora prior to oral or intranasal inoculation. The mice continued to be fed with 250μg/ml kanamycin water for two more days and were inoculated a second time with the recombinant *S. gordonii*. Bacteriological samples were obtained as described herein. The results showed that 12 of the 14 mice were colonized by the modified bacterium. Eight of the 12 colonized mice retained the recombinant bacterium for a minimum of 10 weeks. These results indicate that oral colonization can readily be achieved. Preliminary analysis of sera and bronchoalveolar lavage fluids showed the presence of detectable anti-Pt IgG and SIgA antibodies.

To determine whether the vaccine strain is successfully colonized in the oral cavity, microbiological samples are obtained on day 4 and every 7 days after the oral inoculation until the mice are sacrificed. Samples of unstimulated whole saliva (10μl/mouse) are collected with sterile cotton swabs and plated on mitis-salivarius agar. Colonies of *S. gordonii* are identified by the serological and biochemical criteria of R.R. Falklam, *J. Clin. Microbiol.* 5:184-201, 1977. The isolates that are identified

as *S. gordonii* are tested for reaction to anti-S1 antibodies by ELISA. A certain proportion of these isolates show reactivity with the antibodies, indicating the presence of the recombinant *S. gordonii*. Alternatively, the microbiological samples are examined directly by immunofluorescent microscopy (M.K. Homonylo-McGavin and S.F. Lee, 1996, *supra*). If any isolates have lost the ability to express the S1 fusion protein, the detection by antibodies will not be successful. In this case, DNA probes are made from the S1 sequence and used to hybridize to the DNA of the isolates. Since the parent strain lacks the S1 DNA sequence, positive hybridization indicates the presence of the recombinant *S. gordonii* in the oral cavity that has failed to express the S1 fusion protein, but still retains the heterologous DNA.

Immune responses.

To investigate whether the oral immunization has elicited immune responses to the S1 fragment, sera, saliva, and bronchoalveolar lavage fluids are obtained from the baby mice at two weeks of age. This timing allows for immune responses to develop and also allows the mice to become large enough to ensure that an adequate amount of samples can be obtained for assays. Sera is prepared from blood obtained from the tail vein. Unstimulated saliva (20-40 μl per animal) is obtained by placing a cotton swab in the oral cavity of the animals for about 15 minutes. Saliva is recovered from the cotton swabs by centrifugation using known techniques. If needed, the animals are injected with Carbacol subcutaneously to induce rapid and short (3 to 5 min) salivation with moderate flow (50-100 μl) (N. Deslauriers et al., *Immunol. Investigations* 15:339-349, 1986) without significant dilution of the SIgA concentration in saliva after a single stimulation. Bronchoalveolar lavage fluids are also obtained using the method described by R.D. Shahin et al., *Infect. Immun.* 58:4063-4068, 1990.

These samples are analyzed to determine their titers and specificity against PT by ELISA and Western blotting, respectively. In ELISA, microtiter plates are coated with 100 ng of PT per well and the antibodies (i.e. IgA, IgG, or IgM) are detected by the respective specific antibodies (e.g. goat anti-mouse IgA, alpha chain specific, for

the detection of IgA) (Sigma). To determine the neutralization of PT in these samples, a known CHO cell clustering assay is used as described in S.A. Halperin et al., 1991, *supra*, which is incorporated herein by reference in its entirety.

Additional assays, the ADP- ribosylation (ADPR) assay and leukocytosis-promoting and histamine-sensitizing activity assay, are also performed to assure that the fusion protein is not toxic to the modified organism. For the anti-ADPR activity, the method used by H. Sato et al., 1991, *supra*, is used. Briefly, CHO cells are lysed in a reaction buffer containing EDTA and cell membranes are collected by centrifugation. Native and antibody-reacted PT are incubated with the CHO cell membranes in a reaction buffer containing [^{32}P]NAD, as described by H. Sato et al., 1991, *supra* . The incorporation of [^{32}P]ADP into the transducin present in the cell membrane is measured by liquid scintillation counting. To confirm the incorporation of [^{32}P]ADP into the 41 kDa transducin, membranes are dissolved in 1% SDS-8 M urea and electrophoresed on a 10% polyacrylamide gel. The [^{32}P]ADP ribosylated transducin is visualized by exposing the gel on an X-ray film. For the leukocytosis assay, a known protocol is followed (S.F. Lee et al., 1998, *supra*; Halperin et al., 1991, *supra*). Briefly, native and antibody-reacted PT is injected intraperitoneally into two groups of mice. A third group of mice receive only buffer as the control. Blood is collected from the tail vein three days after injection and the leukocyte counts are determined in a Coulter Counter (S.A. Halperin et al., 1991, *supra*). The following day, mice are challenged with histamine diphosphate (2 mg) and monitored for death. Native PT at 500 ng level has been shown to elevate the leukocyte count 3-fold when compared to mice that received only buffer, and nine of ten mice that received this dose of PT die after the injection of histamine base (J. L. Arciniega et al., *Infect. Immun.* 59:3407-3410, 1991). Monoclonal anti-S1 antibodies neutralize the leukocytosis promoting effect of PT (S.A. Halperin et al., 1991, *supra*; H. Sato et al., *Infect. Immun.* 55:909-915, 1987). Hence, it is shown that the anti-S1 fusion protein antibodies have a neutralizing capability.

Toxin challenge of immunized mice.

When the animals are shown to produce the anti-PT antibodies that confer protective effects by *in vitro* experiments, the mice are challenged with PT. Native PT (500 ng/mouse) in PBS is administered intraperitoneally into the animals. Three days after challenge, the leukocyte count per ml of blood is determined. The following day, mice are subjected to the histamine sensitizing activity assay as described above. Mice that have not been orally immunized with the vaccine bacteria are used as the control. The immunized mice are expected to have a lower leukocyte count than the non-immunized mice and are expected to survive the histamine challenge.

***B. pertussis* aerosol challenge.**

The mouse model of *B. pertussis* infection is a sublethal aerosol infection as described previously (S.A. Halperin et al., 1991, *supra*). At 25 days of age, cohorts of mice (orally immunized or control) are exposed to an aerosol of 5×10^8 CFU/ml of virulent *B. pertussis* (Tohama); this dose induces a non-lethal infection in over 90% of mice (the rest are uninfected). Peak infection occurs two weeks following inoculation, however, all mice recover. Manifestations of the infection are pneumonia and decreased weight gain; however, the animals continue to be active and there is no visible alteration of coat texture. The infection is followed by a daily examination. At two weekly intervals, a cohort of mice are sacrificed, and a quantitative lung count is performed. Additional cohorts are examined at four and eight weeks post-infection. Blood for serology is obtained by cardiac puncture after sacrificing the mice by CO_2 inhalation. Tracheal-bronchial washes are also performed in the sacrificed animals using phosphate-buffered saline. Protective ability of the oral immunization regimen is determined through comparison of quantitative lung bacterial counts. Protection is correlated with antibody titers in blood, saliva, and tracheobronchial washes.

EXAMPLE 3

Recombinant *S. gordonii* expressing polyvalent antigens.

In clinical studies, vaccines containing multiple components of *B. pertussis* are more effective at preventing pertussis than vaccines containing one or two components (E.L. Hewlett, *Pediatr. Infect. Dis. J.* 16: (4 Suppl):S78-84, 1997). Hence, *S. gordonii* expressing multiple *B. pertussis* antigens in addition to the S1 fragment are constructed and tested in the animal infection model described in Examples 1 and 2 above.

Polyvalent *S. gordonii* construction.

Antibodies against S3 subunit have been shown to be protective (S.A. Halperin et al., 1991, *supra*; H. Sato et al., 1991, *supra*). Consequently, part of S3 is cloned into the SpaP-S1 fusion using strategy previously described (S.F. Lee et al., 1998, *supra*). Briefly, a 534 bp fragment from the PT operon carried on pPTX42 (C. Locht and J.M. Keith, *supra*) was PCR amplified using the following primer pair:

5'-TTACCCGGGACCCAACAGGGCGGCGC-3' (SEQ ID NO:3) and

5'-CTCGGTACCGCGATATCGAGGGAAATGCCGGTGA-3'
(SEQ ID NO:4).

This 534 bp DNA fragment codes for a 178-amino acid fragment (amino acid residue #14 - #192) from S3 (mature S3 contains 199 amino acids). The amplicon contains convenient *Kpn*I and *Sma*I restriction sites for in-frame cloning into the *Nru*I site downstream to the S1 fragment in the SpaP-S1 construct. The resulting bivalent construct is introduced into *S. gordonii*. Expression of the S3 fragment is confirmed by Western blotting using an anti-S3 monoclonal antibody (Mab B9, S.A. Halperin, et al., 1991, *supra*) and by immunogold electron microscopy. In Western blots rabbit anti-PT antiserum (1/200) reacts with the S1, SA2 and S3 of PT (1 g, List Biological Laboratories, Inc.), rabbit anti-SpaP-S1 antiserum reacts with the S1 subunit of PT only, but preimmune serum (1/100) obtained from the same rabbits used to raise the

anti-SpaP-S1 serum did not react with PT. In immunogold electron micrographs, the RJMIII cells containing *S. gordonii* expressing SpaP-S1 fusion protein on the cell surface were labeled with gold conjugates after reaction with the monoclonal anti-SpaP antibody and the anti-PT antibodies, respectively. In addition, the amplicon is also fused to *spaP*, creating a monovalent construct for comparison. The parent *S. gordonii* DL-1 cells showed the lack of gold labels following the same treatment.

Fragments of pertactin, fimbriae and FHA are added to the SpaP-S1/S3 as additional antigens. The DNA sequences coding for these antigens are either available from the GenBank or from publications. DNA fragments containing immunogenic epitopes are chosen based on information available from the literature. For pertactin, the C-terminal $(PQP)_5$ repeating sequence has been shown to contain major immunoprotective epitopes (I.G. Charles et al., *Eur. J. Immunol.* 21:1147-1153, 1991), hence it represents an excellent choice for use. For fimbriae, DNA coding for the entire major subunit Fim2 (22.5 kDa) of type 2 fimbriae is used (I. Livey et al., *Mol. Microbiol.* 1:203-209, 1987). A.M. Pearce et al., *Microbiology* 140:205-211, 1994 previously showed that the antigenic epitopes in Fim2 (and Fim3 of type 3 fimbrae) are non-contiguous and may be conformationally dependent. Hence, use of peptide fragments is not preferred. For FHA, the recently identified type I immunodominant domain (ca. 450 amino acid residues in the C terminus of the mature 220 kDa FHA) is used (E. Leininger et al., *J. Infect. Dis.* 175:1473-1431, 1997). This type I domain elicits an immune response in mice and humans (with the disease or after vaccination).

To clone these fragments, primers are designed using known methods to PCR amplify the DNA from the chromosome of *B. pertussis*. Each of the DNA fragments is separately fused to *spaP* initially and tested for expression to ensure the fragment is not toxic to *S. gordonii*. Polyclonal and a monoclonal antibody to FHA have been developed and are used for the detection of FHA fragments expressed in *S. gordonii*. Anti-pertactin and anti-fimbriae antibodies have been obtained from established collaborations with colleagues in other laboratories (e.g. the US FDA Centers for Biologics Evaluation and Research pertussis laboratory).

The final pentavalent fusion protein contains a 179-amino acid (aa) fragment of S1, a 178-aa S3 fragment, a 80-aa fragment of pertactin, a 205-aa Fim2 fragment, and a 450-aa FHA fragment. Alternatively, a less bulky version of the construct is obtained by deleting part of the *spaP* sequence to limit the final fusion protein to not more than 259 kDa. Because *S. gordonii* produces a surface multi-functional adhesin (CshA) of 259 kDa (R. McNab et al., *Mol. Microbiol.* 14:743-754, 1994) and can surface-express the 187 kDa SpaP-S1 fusion protein, polyvalent proteins of this size range are also correctly processed and surface-localized in this bacterium.

To monitor whether the immunogenic epitopes of the recombinant antigens are properly presented, recognition of the recombinant antigens by antibodies to known functional sites with secondary structure on the native pertussis antigen is tested by ELISA using a known method (S.F. Lee, et al., 1998, *supra*) and antibodies already developed for the epitopes as described above and as available from US FDA Centers for Biologics Evaluation and Research pertussis laboratory. In this assay, the recombinant *S. gordonii* whole-cell is used as antigen source in one study, and in another purified recombinant antigens are used. The recombinant antigens are purified from the culture supernatant fluid or from the cell walls by mutanolysin-lysozyme treatment (M.K. Homonylo-McGavin and S.F. Lee, 1996, *supra*) using affinity column chromatography with anti-SpaP antibody-coupled Protein A Sepharose, or alternatively, by conventional column chromatography. Further monitoring is performed by a competitive ELISA assay as described by E. Harlow and D. Lane, *Antibodies: A laboratory manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988. In brief, in the competitive ELISA, after coating the microtiter plates with whole-cells or purified recombinant antigen, the plates are reacted simultaneously with a rabbit antibody to the recombinant antigen and the known monoclonal antibody for competitive binding. If the sites of interaction are identical or overlapping, the known antibody competes, and the amount of anti-recombinant antigen antibody bound to the antigen is lowered. If the known antibody is in excess, no anti-recombinant antigen antibody binds. The results from these

assays indicate whether the recombinant antigen maintains a secondary structure similar to the native antigen.

Protective effects of the polyvalent *S. gordonii*.

The immunogenicity of the constructs is tested by assaying antibody titers after immunizing animals with the recombinant *S. gordonii*. Heat-killed recombinant *S. gordonii* is used to immunize rabbits or mice, as described previously (S. F. Lee et al., 1998, *supra*). Sera obtained is tested for reactivity with the native antigens (available commercially or from other laboratories) by ELISA and Western blotting. The mouse infection model described in Example 2 above is used to test whether the polyvalent strain is any better than the monovalent vaccine in protecting the animals from *B. pertussis* infection.

Efficacy of recombinant *S. gordonii* against *B. pertussis* infection.

This experiment compares the effectiveness of the invention live oral vaccines to that of the conventional acellular pertussis vaccines for protecting mice from *B. pertussis* infection. Cohorts of mice are immunized with a commercial acellular vaccine via intraperitoneal injections using a product with a similar antigen profile (e.g. the acellular vaccine containing PT, FHA, and pertactin); the recommended human dose (0.5 ml) is used and a two to three dose primary series is given at two week intervals. The second cohort of mice is orally immunized with the recombinant *S. gordonii* strain described above expressing similar antigens (i.e. S1, S3, FHA and pertactin fragments). Once immune responses have been elicited after immunization, the two groups of mice are challenged with *B. pertussis* as described in Example 2 above. The results obtained indicate the effectiveness of the polyvalent *S. gordonii* vaccine in comparison to the commercial acellular vaccine.

It will be apparent to those skilled in the art that various changes may be made in the invention without departing from the spirit and scope thereof, and therefore, the invention encompasses embodiments in addition to those specifically disclosed in the specification, but only as indicated in the appended claims.

WHAT IS CLAIMED IS:

1. A composition for the stimulation of protection against infection by at least one pathogen, said composition comprising a live commensal oral organism genetically modified so as to express a plurality of immunogenic fragments of said pathogen.

2. A composition according to claim 1 wherein said plurality of immunogenic fragments are derived from the same mucosal pathogen.

3. A composition according to claim 1 wherein said plurality of immunogenic fragments are derived from more than one pathogen.

4. A composition according to claim 1 wherein said pathogen is *Bordetella pertussis*, Respiratory Syncytial Virus (RSV), poliovirus, *Mycoplasma pneumoniae*, meningococcus, pneumococcus, rotavirus, influenza, parainfluenza, *Corynebacterium diphtheriae*, *Clostridium tetani*, hepatitis B virus, Neisseria gonorrhoeae, non-typeable Haemophilus influenzae, Chlamydia pneumoniae, Chlamydia trachomatis, Moraxella catarrhalis, or a combination of two or more thereof.

5. A composition according to claim 1 wherein said pathogen is *Bordetella pertussis*.

6. A composition according to claim 5 wherein said immunogenic fragment is derived from the pertussis toxin.

7. A composition according to claim 6 wherein said immunogenic fragment of the pertussis toxin comprises the N-terminal 179 amino acids of the S1 subunit of the pertussis toxin.

8. A composition according to claim 5 wherein said immunogenic fragment is derived from one or more of the pertussis toxin, filamentous hemagglutinin, pertactin and fimbriae.

9. A composition according to claim 1 wherein said commensal oral organism is a *Streptococcus*.

10. A composition according to claim 9 wherein said commensal oral organism is *Streptococcus gordonii, Streptococcus salivarius* or *Streptococcus mitis.*

11. A composition according to claim 10 wherein said genetic modification comprises transformation of said *Streptococcus gordonii* with a vector encoding the surface protein antigen P1 of *Streptococcus mutans,* and wherein the sequence encoding said surface protein antigen is modified by insertion of sequence encoding said immunogenic fragment therein.

12. A composition according to claim 1 wherein said organism is further modified so as to express at least one mucosal adjuvant.

13. A composition according to calim 1 wherein said composition further comprises at least one immunological adjuvant.

14. A method for prophylactically treating a host against infection by a pathogen, said method comprising orally and/or intranasally administering to said host an effective amount of a composition according to claim 1.

15. A method according to claim 14 wherein said plurality of immunogenic fragments are derived from the same pathogen.

16. A method according to claim 14 wherein said plurality of immunogenic fragments are derived from more than one pathogen.

17. A method according to claim 14 wherein said pathogen is *Bordetella pertussis*, Respiratory Syncytial Virus, poliovirus, *Mycoplasma pneumoniae*, meningococcus, pneumococcus, rotavirus, influenza, parainfluenza, *Corynebacterium diphtheriae*, *Clostridium tetani*, Neisseria gonorrhoeae, non-typeable Haemophilus influenzae, Chlamydia pneumoniae, Chlamydia trachomatis, Moraxella catarrhalis, hepatitis B virus, or a combination of two or more thereof.

18. A method according to claim 17 wherein said pathogen is *Bordetella pertussis*.

19. A method according to claim 18 wherein said immunogenic fragment is derived from the pertussis toxin.

20. A method according to claim 19 wherein said immunogenic fragment of the pertussis toxin comprises the N-terminal 179 amino acids of the S1 subunit of the pertussis toxin.

21. A method according to claim 18 wherein said immunogenic fragment is derived from one or more of the pertussis toxin, filamentous hemagglutinin, pertactin and fimbriae.

22. A method according to claim 14 wherein said commensal oral organism is *Streptococcus*.

23. A method according to claim 14 wherein said organism is further modified so as to express at least one mucosal adjuvant.

24. A method according to claim 14 wherein said composition further comprises at least one immunological adjuvant.

25. A method for chronic immunization of a host against infection by a pathogen, said method comprising orally and/or intranasally administering to said host an effective amount of a composition according to claim 1.

26. A method according to claim 25 wherein said plurality of immunogenic fragments are derived from the same pathogen.

27. A method according to claim 25 wherein said plurality of immunogenic fragments are derived from more than one pathogen.

28. A method according to claim 25 wherein said pathogen is *Bordetella pertussis*, Respiratory Syncytial Virus, poliovirus, *Mycoplasma pneumoniae*, meningococcus, pneumococcus, rotavirus, influenza, parainfluenza, *Corynebacterium diphtheriae*, *Clostridium tetani*, Neisseria gonorrhoeae, non-typeable Haemophilus influenzae, Chlamydia pneumoniae, Chlamydia trachomatis, Moraxella catarrhalis, hepatitis B virus, or a combination of two or more thereof.

29. A method according to claim 28 wherein said pathogen is *Bordetella pertussis*.

30. A method according to claim 29 wherein said immunogenic fragment is derived from the pertussis toxin.

31. A method according to claim 30 wherein said immunogenic fragment of the pertussis toxin comprises the N-terminal 179 amino acids of the S1 subunit of the pertussis toxin.

32. A method according to claim 29 wherein said immunogenic fragment is derived from one or more of the pertussis toxin, filamentous hemagglutinin, pertactin and fimbriae.

33. A method according to claim 25 wherein said commensal oral organism is *Streptococcus*.

34. A method according to claim 25 wherein said organism is further modified so as to express at least one mucosal adjuvant.

35. A method according to claim 25 wherein said composition further comprises at least one immunological adjuvant.

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(57) Abstract

In accordance with the present invention, there are provided compositions comprising a live oral commensal bacteria, such as a streptococci, that has been genetically modified to express immunogenic fragment(s) of one or more pathogens, such as a mucosal pathogen like B. pertussis, which causes the disease known as whooping cough. The modified organisms are administered orally and/or intranasally. Once an infection of the modified commesal bacteria is established in the oral cavity of the host, the modified organism will continue to express the immunogenic pathogenic fragment(s) so long as the infection persists. Since such oral commensal bacteria generally persist in the oral cavity of humans and other susceptible vertebrates throughout life, the need for booster immunizations is minimized or reduced, providing the host protection against the pathogen throughout life. As native oral commensal organisms are spread from mother to infant as well as between adults by daily contact, invention compositions and methods will be particularly useful for providing immunity in undeveloped countries where conventional vaccines are too expensive to be effectively used.

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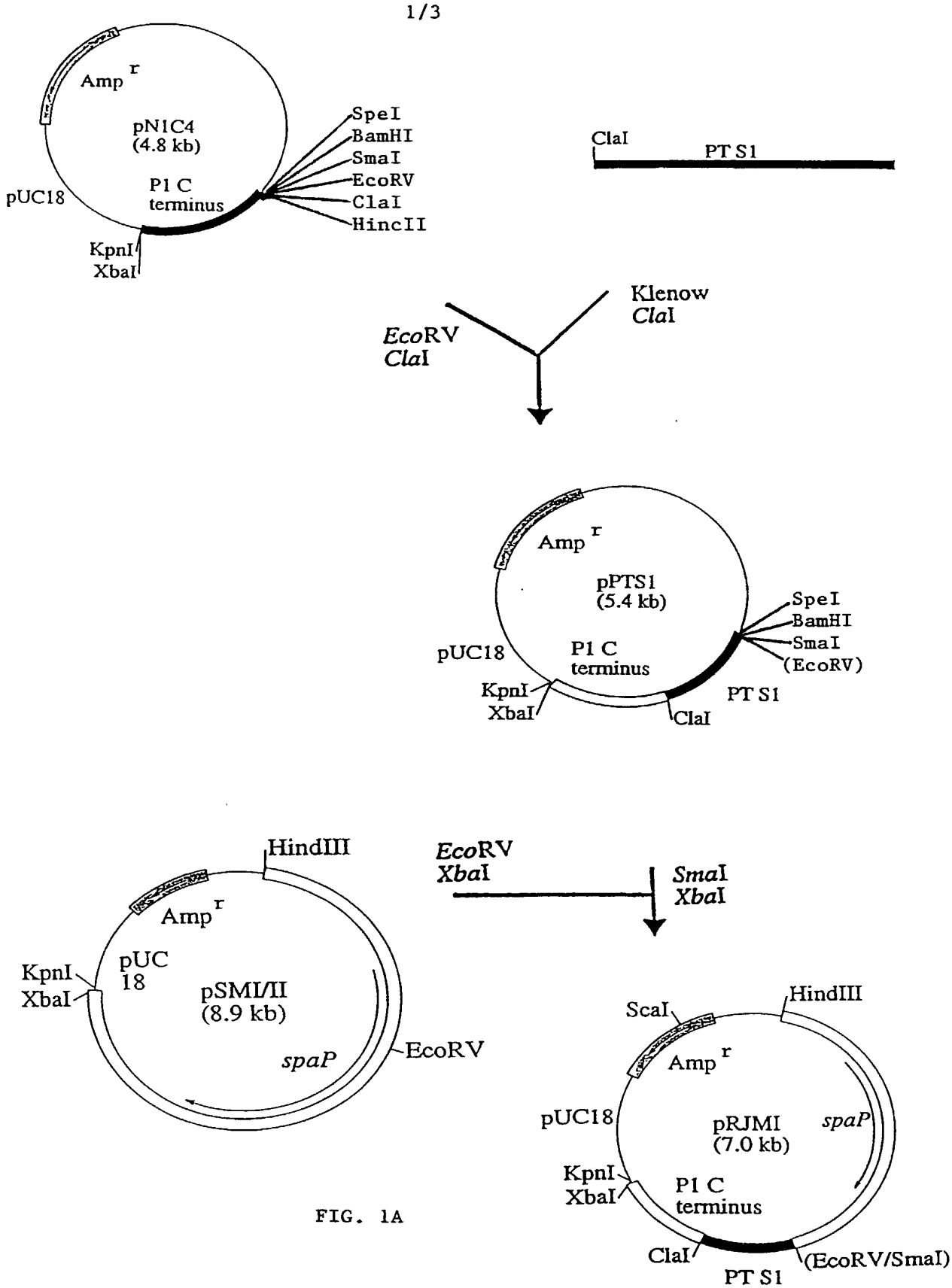
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FIG. 1A

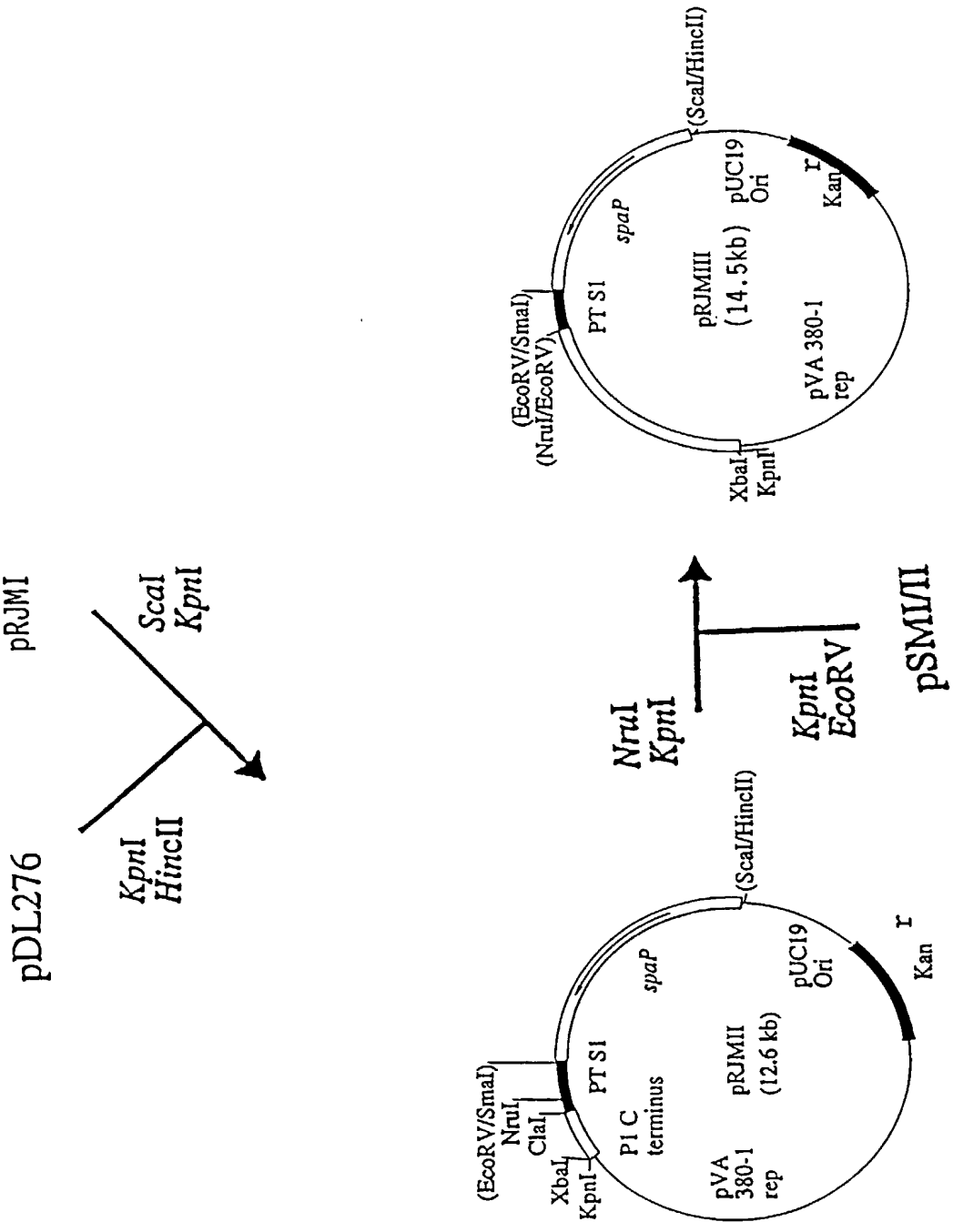
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FIG. 1B

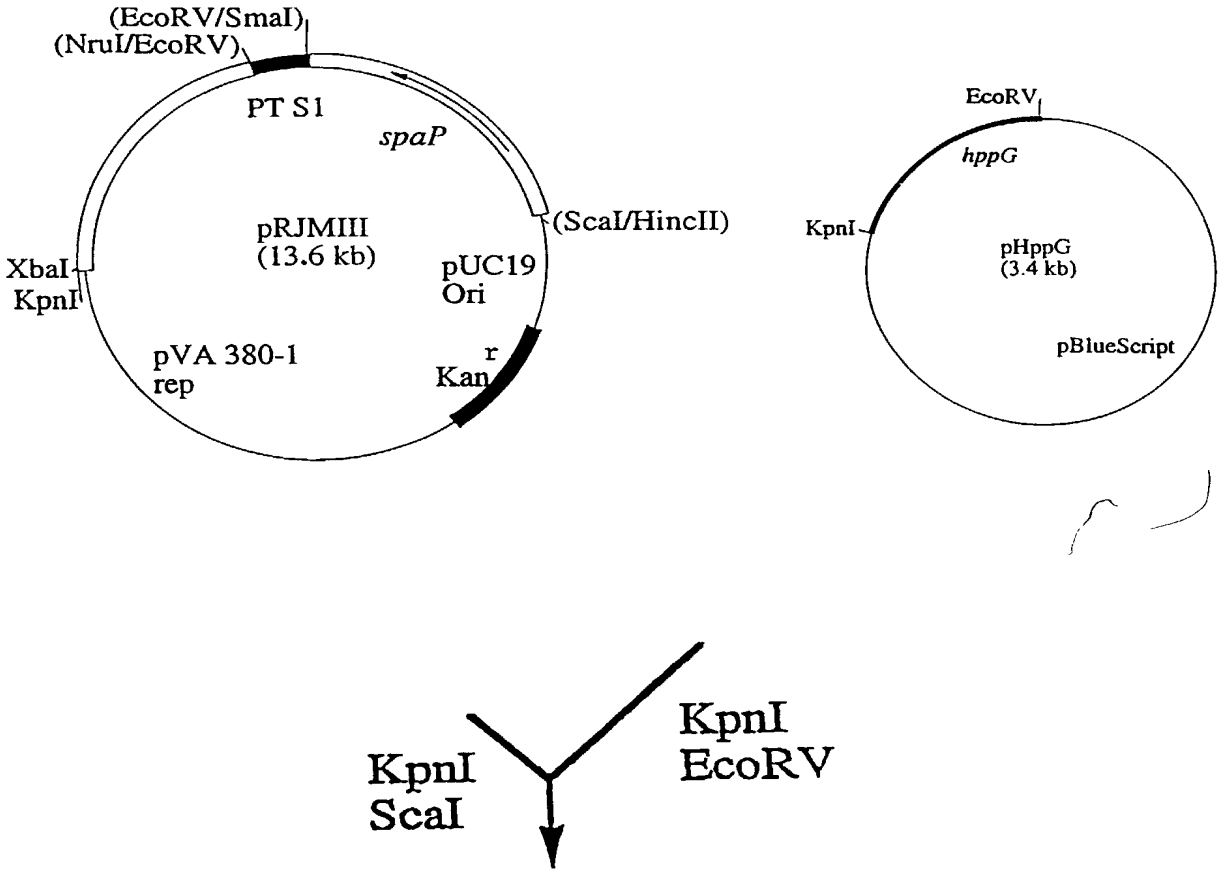
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FIG. 2

Atty. Dkt. No. DALHO1340-1
(028614-1303)

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I HEREBY DECLARE:

THAT my residence, post office address, and citizenship are as stated below next to my name;

THAT I believe I am the original, first, and sole inventor (if only one inventor is named below) or an original, first, and joint inventor (if plural inventors are named below or in an attached Declaration) of the subject matter which is claimed and for which a patent is sought on the invention entitled

GENETICALLY ENGINEERED ORAL COMMENSAL ORGANISMS AS VACCINES

(Attorney Docket No. DALHO1340-1)

the specification of which (check one)

____ is attached hereto.

_X_ was filed on 10/23/2001 as United States Application Number or PCT International Application Number 10/019,453 and was amended on _____ (if applicable).

THAT I do not know and do not believe that the same invention was ever known or used by others in the United States of America, or was patented or described in any printed publication in any country, before I (we) invented it;

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THAT I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment specifically referred to above;

THAT I believe that the above-identified specification contains a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention, and sets forth the best mode contemplated by me of carrying out the invention; and

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THAT I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

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US00/10954	P.C.T.	04/21/2000	YES	

I HEREBY CLAIM the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

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10/019,453		10/23/2001	

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to have full power to prosecute this application and any continuations, divisions, reissues, and reexaminations thereof, to receive the patent, and to transact all business in the United States Patent and Trademark Office connected therewith.

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I UNDERSTAND AND AGREE THAT the foregoing attorneys and agents appointed by me to prosecute this application do not personally represent me or my legal interests, but instead represent the interests of the legal owner(s) of the invention described in this application.

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